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(54) Title: HLA BINDING PEPTIDES AND THEIR USES (57) Abstract The present invention provides peptide compositions capable of specifically binding selected MHC alleles and inducing T cell activation in T cells restricted by the MHC allele. The peptides are useful to elicit an immune response against a desired antigen.		

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HLA BINDING PEPTIDES AND THEIR USES

The present application is a continuation in part of USSN 08/027,746 which is a continuation in part of USSN 07/926,666, which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

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The present invention relates to compositions and methods for preventing, treating or diagnosing a number of pathological states such as viral diseases and cancers. In particular, it provides novel peptides capable of binding selected major histocompatibility complex (MHC) molecules and inducing an immune response.

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MHC molecules are classified as either Class I or Class II molecules. Class II MHC molecules are expressed primarily on cells involved in initiating and sustaining immune responses, such as T lymphocytes, B lymphocytes, macrophages, etc. Class II MHC molecules are recognized by helper T lymphocytes and induce proliferation of helper T lymphocytes and amplification of the immune response to the particular immunogenic peptide that is displayed. Class I MHC molecules are expressed on almost all nucleated cells and are recognized by cytotoxic T lymphocytes (CTLs), which then destroy the antigen-bearing cells. CTLs are particularly important in tumor rejection and in fighting viral infections. The CTL recognizes the antigen in the form of a peptide fragment bound to the MHC class I molecules rather than the intact foreign antigen itself. The antigen must normally be endogenously synthesized by the cell, and a portion of the protein antigen is degraded into small peptide fragments in the cytoplasm. Some of these small peptides translocate into a pre-Golgi compartment and interact with class I heavy chains to facilitate proper folding and association with the subunit $\beta 2$ microglobulin. The peptide-MHC class I complex is then routed to the cell surface for expression and potential recognition by specific CTLs.

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Investigations of the crystal structure of the human MHC class I molecule, HLA-A2.1, indicate that a peptide binding groove is created by the folding of the $\alpha 1$ and $\alpha 2$ domains of the class I heavy chain (Bjorkman et al., Nature 329:506 (1987)). In these investigations, however, the identity of peptides bound to the groove was not determined.

Buus et al., Science 242:1065 (1988) first described a method for acid elution of bound peptides from MHC.

Subsequently, Rammensee and his coworkers (Falk et al., Nature 351:290 (1991)) have developed an approach to characterize naturally processed peptides bound to class I molecules.

Other investigators have successfully achieved direct amino acid sequencing of the more abundant peptides in various HPLC fractions by conventional automated sequencing of peptides eluted from class I molecules of the B type (Jardetzky, et al., Nature 353:326 (1991)) and of the A2.1 type by mass spectrometry (Hunt, et al., Science 225:1261 (1992)). A review of the characterization of naturally processed peptides in MHC Class I has been presented by Rötzschke and Falk (Rötzschke and Falk, Immunol. Today 12:447 (1991)).

Sette et al., Proc. Natl. Acad. Sci. USA 86:3296 (1989) showed that MHC allele specific motifs could be used to predict MHC binding capacity. Schaeffer et al., Proc. Natl. Acad. Sci. USA 86:4649 (1989) showed that MHC binding was related to immunogenicity. Several authors (De Bruijn et al., Eur. J. Immunol., 21:2963-2970 (1991); Pamer et al., 1991 Nature 353:852-955 (1991)) have provided preliminary evidence that class I binding motifs can be applied to the identification of potential immunogenic peptides in animal models. Class I motifs specific for a number of human alleles of a given class I isotype have yet to be described. It is desirable that the combined frequencies of these different alleles should be high enough to cover a large fraction or perhaps the majority of the human outbred population.

Despite the developments in the art, the prior art has yet to provide a useful human peptide-based vaccine or therapeutic agent based on this work. The present invention provides these and other advantages.

SUMMARY OF THE INVENTION

The present invention provides compositions comprising immunogenic peptides having binding motifs for MHC Class I molecules. The immunogenic peptides are typically
5 between about 8 and about 11 residues and comprise conserved residues involved in binding proteins encoded by the appropriate MHC allele. A number of allele specific motifs have been identified.

For instance, the motif for HLA-A3.2 comprises from
10 the N-terminus to C-terminus a first conserved residue of L, M, I, V, S, A, T and F at position 2 and a second conserved residue of K, R or Y at the C-terminal end. Other first conserved residues are C, G or D and alternatively E. Other second conserved residues are H or F. The first and second
15 conserved residues are preferably separated by 6 to 7 residues.

The motif for HLA-A1 comprises from the N-terminus to the C-terminus a first conserved residue of T, S or M, a second conserved residue of D or E, and a third conserved
20 residue of Y. Other second conserved residues are A, S or T. The first and second conserved residues are adjacent and are preferably separated from the third conserved residue by 6 to 7 residues. A second motif consists of a first conserved residue of E or D and a second conserved residue of Y where
25 the first and second conserved residues are separated by 5 to 6 residues.

The motif for HLA-A11 comprises from the N-terminus to the C-terminus a first conserved residue of T or V at position 2 and a C-terminal conserved residue of K. The first
30 and second conserved residues are preferably separated by 6 or 7 residues.

The motif for HLA-A24.1 comprises from the N-terminus to the C-terminus a first conserved residue of Y, F or W at position 2 and a C-terminal conserved residue of F, I,
35 W, M or L. The first and second conserved residues are preferably separated by 6 to 7 residues.

Epitopes on a number of potential target proteins can be identified in this manner. Examples of suitable

antigens include prostate specific antigen (PSA), hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, malignant melanoma antigen (MAGE-1) Epstein-Barr virus antigens, human immunodeficiency type-1 virus (HIV1) and
5 papilloma virus antigens. The peptides are thus useful in pharmaceutical compositions for both in vivo and ex vivo therapeutic and diagnostic applications.

Definitions

10 The term "peptide" is used interchangeably with "oligopeptide" in the present specification to designate a series of residues, typically L-amino acids, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of adjacent amino acids. The
15 oligopeptides of the invention are less than about 15 residues in length and usually consist of between about 8 and about 11 residues, preferably 9 or 10 residues.

An "immunogenic peptide" is a peptide which comprises an allele-specific motif such that the peptide will
20 bind the MHC allele and be capable of inducing a CTL response. Thus, immunogenic peptides are capable of binding to an appropriate class I MHC molecule and inducing a cytotoxic T cell response against the antigen from which the immunogenic peptide is derived.

25 A "conserved residue" is an amino acid which occurs in a significantly higher frequency than would be expected by random distribution at a particular position in a peptide motif. Typically a conserved residue is one at which the immunogenic peptide may provide a contact point with the MHC
30 molecule. One to three, preferably two, conserved residues within a peptide of defined length defines a motif for an immunogenic peptide. These residues are typically in close contact with the peptide binding groove, with their side chains buried in specific pockets of the groove itself.
35 Typically, an immunogenic peptide will comprise up to three conserved residues, more usually two conserved residues.

As used herein, "negative binding residues" are amino acids which if present at certain positions will result

in a peptide being a nonbinder or poor binder and in turn fail to induce a CTL response despite the presence of the appropriate conserved residues within the peptide.

5 The term "motif" refers to the pattern of residues in a peptide of defined length, usually about 8 to about 11 amino acids, which is recognized by a particular MHC allele. The peptide motifs are typically different for each human MHC allele and differ in the pattern of the highly conserved residues.

10 The binding motif for an allele can be defined with increasing degrees of precision. In one case, all of the conserved residues are present in the correct positions in a peptide and there are no negative binding residues present.

15 The phrases "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. Thus, the peptides of this invention do not contain materials normally associated with their in situ environment, e.g., MHC I molecules on antigen presenting cells. Even where
20 a protein has been isolated to a homogenous or dominant band, there are trace contaminants in the range of 5-10% of native protein which co-purify with the desired protein. Isolated peptides of this invention do not contain such endogenous co-purified protein.

25 The term "residue" refers to an amino acid or amino acid mimetic incorporated in an oligopeptide by an amide bond or amide bond mimetic.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a flow diagram of an HLA-A purification scheme.

Fig. 2 is an SDS-PAGE analysis of affinity purified. HLA-A3.2 from the cell line EHM using an affinity column prepared with the mAb GAP A3 coupled to protein A-Sepharose.

Lane 1 - Molecular weight standards.

Lane 2 - A3.2 acid eluate

Lane 3 - A3.2 a second acid eluate

Lane 4 - Base elution #1

Lane 5 - Base elution #2

Lane 6 - Concentrated base elution 1

Lane 7 - Concentrated base elution 2

Lane 8 - BSA - 10 μ g

Lane 9 - BSA - 3 μ g

Lane 10 - BSA - 1 μ g

Fig. 3 shows reverse phase high performance liquid chromatography (RP-HPLC) separation of HLA-A3 acid eluted peptides.

Fig. 4 shows binding of a radioactively labeled peptide of the invention to MHC molecules as measured by the bound radioactivity.

Fig. 5 shows inhibition of binding of a peptide of the invention to MHC molecules in the presence of three peptides [HBc 18-27 (924.07), a Prostate Specific Antigen peptide (939.01), and HIV nef 73-82 (940.03)].

Fig. 6 shows the dependency of the binding on MHC concentration in the presence or absence of β_2 microglobulin.

Fig. 7 shows dose dependent inhibition of binding with the addition of unlabeled peptide.

Fig. 8 Scatchard Analysis of binding to MHC A11 confirming an apparent K_D of 6nM.

Fig. 9 shows the binding of a radioactively labeled peptide of the invention to MHC A1 as measured by bound reactivity.

Fig. 10 shows dose dependent inhibition of binding with the addition of unlabeled peptide.

Fig. 11 Scatchard Analysis of binding to MHC A1 confirming an apparent K_D of 21nM.

Fig. 12 shows the binding of two peptides of this invention as a function of MHC A24 concentration as measured by % bound reactivity.

Fig. 13 shows the dose dependent inhibition of binding to MHC A24 with the addition of unlabeled peptides.

Figs. 14(a) and 14(b) show the Scatchard Analysis of binding to MHC A24 of the two peptides confirming a K_D of 30 and 60nM, respectively.

Fig. 15 shows the effect on MHC class I molecules of β_2 microglobulin and a peptide of choice on acid-stripped PHA blasts.

Fig. 16 shows CTL induction using GC43 A2.1 responders and autologous acid-stripped PBMCs or PHA blasts loaded with the 777.03-924.07-927.32 peptide pool.

Fig. 17 shows CTL induction using X351 or X355 A2.1 responders and autologous acid stripped PBMCs or PHA blasts as stimulators after loading with the 1044.04-1044.05-1044.06 peptide pool.

Fig. 18 shows CTL induction using GC49 A2.1 responders and Autologous Acid stripped PHA blasts as stimulators after loading with 939.03 peptide.

Fig. 19 shows CTL induction using GC66 A1 responders and autologous acid stripped PBMCs as stimulators after loading of peptide 938.01.

Fig. 20 illustrates the lysis of peptide sensitized targets and endogenous targets following stimulation with SAC-I activated PBMCs loaded with a MAGE 3 peptide.

Fig. 21 shows a comparison of the acid strip loading with the cold temperature incubation.

Fig. 22 shows a CTL response to an immunogenic peptide for MAGE/A11.

Fig. 23 shows a CTL response to an immunogenic peptide for HIV/A3.

Fig. 24 shows a CTL response to an immunogenic peptide for HCV/A3.

Fig. 25 shows a CTL response to an immunogenic peptide for HBV/A1.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 The present invention relates to the determination of allele-specific peptide motifs for human Class I MHC (sometimes referred to as HLA) allele subtypes. These motifs are then used to define T cell epitopes from any desired antigen, particularly those associated with human viral
10 diseases or cancers, for which the amino acid sequence of the potential antigen targets is known.

 Epitopes on a number of potential target proteins can be identified in this manner. Examples of suitable antigens include prostate specific antigen (PSA), hepatitis B
15 core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, melanoma antigens (e.g., MAGE-1), human immunodeficiency virus (HIV) antigens and human papilloma virus (HPV) antigens.

 Peptides comprising these epitopes are synthesized
20 and then tested for their ability to bind to the appropriate MHC molecules in assays using, for example, purified class I molecules and radioiodinated peptides and/or cells expressing empty class I molecules by, for instance, immunofluorescent staining and flow microfluorimetry, peptide-dependent class I
25 assembly assays, and inhibition of CTL recognition by peptide competition. Those peptides that bind to the class I molecule are further evaluated for their ability to serve as targets for CTLs derived from infected or immunized individuals, as well as for their capacity to induce primary in vitro or in
30 vivo CTL responses that can give rise to CTL populations capable of reacting with virally infected target cells or tumor cells as potential therapeutic agents.

 The MHC class I antigens are encoded by the HLA-A, B, and C loci. HLA-A and B antigens are expressed at the cell
35 surface at approximately equal densities, whereas the expression of HLA-C is significantly lower (perhaps as much as 10-fold lower). Each of these loci have a number of alleles.

The peptide binding motifs of the invention are relatively specific for each allelic subtype.

For peptide-based vaccines, the peptides of the present invention preferably comprise a motif recognized by an MHC I molecule having a wide distribution in the human population. Since the MHC alleles occur at different frequencies within different ethnic groups and races, the choice of target MHC allele may depend upon the target population. Table 1 shows the frequency of various alleles at the HLA-A locus products among different races. For instance, the majority of the Caucasoid population can be covered by peptides which bind to four HLA-A allele subtypes, specifically HLA-A2.1, A1, A3.2, and A24.1. Similarly, the majority of the Asian population is encompassed with the addition of peptides binding to a fifth allele HLA-A11.2.

TABLE 1

	<u>A Allele/Subtype</u>	<u>N(69)*</u>	<u>A(54)</u>	<u>C(502)</u>
5	A1	10.1(7)	1.8(1)	27.4(138)
	A2.1	11.5(8)	37.0(20)	39.8(199)
	A2.2	10.1(7)	0	3.3(17)
	A2.3	1.4(1)	5.5(3)	0.8(4)
	A2.4	-	-	-
10	A2.5	-	-	-
	A3.1	1.4(1)	0	0.2(0)
	A3.2	5.7(4)	5.5(3)	21.5(108)
	A11.1	0	5.5(3)	0
	A11.2	5.7(4)	31.4(17)	8.7(44)
15	A11.3	0	3.7(2)	0
	A23	4.3(3)	-	3.9(20)
	A24	2.9(2)	27.7(15)	15.3(77)
	A24.2	-	-	-
	A24.3	-	-	-
20	A25	1.4(1)	-	6.9(35)
	A26.1	4.3(3)	9.2(5)	5.9(30)
	A26.2	7.2(5)	-	1.0(5)
	A26V	-	3.7(2)	-
	A28.1	10.1(7)	-	1.6(8)
25	A28.2	1.4(1)	-	7.5(38)
	A29.1	1.4(1)	-	1.4(7)
	A29.2	10.1(7)	1.8(1)	5.3(27)
	A30.1	8.6(6)	-	4.9(25)
	A30.2	1.4(1)	-	0.2(1)
30	A30.3	7.2(5)	-	3.9(20)
	A31	4.3(3)	7.4(4)	6.9(35)
	A32	2.8(2)	-	7.1(36)
	Aw33.1	8.6(6)	-	2.5(13)
	Aw33.2	2.8(2)	16.6(9)	1.2(6)
35	Aw34.1	1.4(1)	-	-
	Aw34.2	14.5(10)	-	0.8(4)
	Aw36	5.9(4)	-	-

40 Table compiled from B. DuPont, Immunobiology of HLA, Vol. I, Histocompatibility Testing 1987, Springer-Verlag, New York 1989.

45 * N - negroid; A = Asian; C = caucasoid. Numbers in parenthesis represent the number of individuals included in the analysis.

50 The nomenclature used to describe peptide compounds follows the conventional practice wherein the amino group is presented to the left (the N-terminus) and the carboxyl group to the right (the C-terminus) of each amino acid residue. In the formulae representing selected specific embodiments of the present invention, the amino- and carboxyl-terminal groups, although not specifically shown, are in the form they would

assume at physiologic pH values, unless otherwise specified. In the amino acid structure formulae, each residue is generally represented by standard three letter or single letter designations. The L-form of an amino acid residue is represented by a capital single letter or a capital first letter of a three-letter symbol, and the D-form for those amino acids is represented by a lower case single letter or a lower case three letter symbol. Glycine has no asymmetric carbon atom and is simply referred to as "Gly" or G.

The procedures used to identify peptides of the present invention generally follow the methods disclosed in Falk et al., Nature 351:290 (1991), which is incorporated herein by reference. Briefly, the methods involve large-scale isolation of MHC class I molecules, typically by immunoprecipitation or affinity chromatography, from the appropriate cell or cell line. Examples of other methods for isolation of the desired MHC molecule equally well known to the artisan include ion exchange chromatography, lectin chromatography, size exclusion, high performance ligand chromatography, and a combination of all of the above techniques.

A large number of cells with defined MHC molecules, particularly MHC Class I molecules, are known and readily available. For example, human EBV-transformed B cell lines have been shown to be excellent sources for the preparative isolation of class I and class II MHC molecules. Well-characterized cell lines are available from private and commercial sources, such as American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," 6th edition (1988) Rockville, Maryland, U.S.A.); National Institute of General Medical Sciences 1990/1991 Catalog of Cell Lines (NIGMS) Human Genetic Mutant Cell Repository, Camden, NJ; and ASHI Repository, Bingham and Women's Hospital, 75 Francis Street, Boston, MA 02115. Table 2 lists some B cell lines suitable for use as sources for HLA-A alleles. All of these cell lines can be grown in large batches and are therefore useful for large scale production of MHC molecules. One of skill will recognize that these are merely exemplary cell lines and that

many other cell sources can be employed. Similar EBV B cell lines homozygous for HLA-B and HLA-C could serve as sources for HLA-B and HLA-C alleles, respectively.

TABLE 2

HUMAN CELL LINES (HLA-A SOURCES)

5		
	HLA-A allele	B cell line
10	A1	MAT COX (9022) STEINLIN (9087)
15	A2.1	JY
20	A3.2	EHM (9080) HO301 (9055)GM3107
25	A24.1	KT3(9107),TISI (9042)
30	A11	BVR (GM6828A) WT100 (GM8602),WT52 (GM8603)
35		

In the typical case, immunoprecipitation is used to isolate the desired allele. A number of protocols can be used, depending upon the specificity of the antibodies used. For example, allele-specific mAb reagents can be used for the affinity purification of the HLA-A, HLA-B, and HLA-C molecules. Several mAb reagents for the isolation of HLA-A molecules are available (Table 3). Thus, for each of the targeted HLA-A alleles, reagents are available that may be used for the direct isolation of the HLA-A molecules. Affinity columns prepared with these mAbs using standard techniques are successfully used to purify the respective HLA-A allele products.

In addition to allele-specific mAbs, broadly reactive anti-HLA-A, B, C mAbs, such as W6/32 and B9.12.1, and

one anti-HLA-B, C mAb, B1.23.2, could be used in alternative affinity purification protocols as described in the example section below.

TABLE 3

ANTIBODY REAGENTS

10	anti-HLA	Name
15		
	HLA-A1	12/18
20	HLA-A3	GAPA3 (ATCC, HB122)
	HLA-11,24.1	A11.1M (ATCC, HB164)
25	HLA-A,B,C	W6/32 (ATCC, HB95)
	monomorphic	B9.12.1 (INSERM-CNRS)
	HLA-B,C	B.1.23.2 (INSERM-CNRS)
30	monomorphic	

35 The peptides bound to the peptide binding groove of the isolated MHC molecules are eluted typically using acid treatment. Peptides can also be dissociated from class I molecules by a variety of standard denaturing means, such as heat, pH, detergents, salts, chaotropic agents, or a
40 combination thereof.

 Peptide fractions are further separated from the MHC molecules by reversed-phase high performance liquid chromatography (HPLC) and sequenced. Peptides can be separated by a variety of other standard means well known to
45 the artisan, including filtration, ultrafiltration, electrophoresis, size chromatography, precipitation with specific antibodies, ion exchange chromatography, isoelectrofocusing, and the like.

Sequencing of the isolated peptides can be performed according to standard techniques such as Edman degradation (Hunkapiller, M.W., et al., Methods Enzymol. 91, 399 [1983]).

Other methods suitable for sequencing include mass

5 spectrometry sequencing of individual peptides as previously described (Hunt, et al., Science 225:1261 (1992), which is incorporated herein by reference). Amino acid sequencing of bulk heterogenous peptides (e.g., pooled HPLC fractions) from different class I molecules typically reveals a characteristic
10 sequence motif for each class I allele.

Definition of motifs specific for different class I alleles allows the identification of potential peptide epitopes from an antigenic protein whose amino acid sequence is known. Typically, identification of potential peptide
15 epitopes is initially carried out using a computer to scan the amino acid sequence of a desired antigen for the presence of motifs. The epitopic sequences are then synthesized. The capacity to bind MHC Class molecules is measured in a variety of different ways. One means is a Class I molecular binding
20 assay as described in Example 10, below. Other alternatives described in the literature include inhibition of antigen presentation (Sette, et al., J. Immunol. 141:3893 (1991), in vitro assembly assays (Townsend, et al., Cell 62:285 (1990), and FACS based assays using mutated cells, such as RMA.S
25 (Melief, et al., Eur. J. Immunol. 21:2963 [1991]).

Next, peptides that test positive in the MHC class I binding assay are assayed for the ability of the peptides to induce specific CTL responses in vitro. For instance, antigen-presenting cells that have been incubated with a
30 peptide can be assayed for the ability to induce CTL responses in responder cell populations. Antigen-presenting cells can be normal cells such as peripheral blood mononuclear cells or dendritic cells (Inaba, et al., J. Exp. Med. 166:182 (1987); Boog, Eur. J. Immunol. 18:219 [1988]).

35 Alternatively, mutant mammalian cell lines that are deficient in their ability to load class I molecules with internally processed peptides, such as the mouse cell lines RMA-S (Kärre, et al., Nature, 319:675 (1986); Ljunggren, et

al., Eur. J. Immunol. 21:2963-2970 (1991)), and the human somatic T cell hybridoma, T-2 (Cerundolo, et al., Nature 345:449-452 (1990)) and which have been transfected with the appropriate human class I genes are conveniently used, when
5 peptide is added to them, to test for the capacity of the peptide to induce in vitro primary CTL responses. Other eukaryotic cell lines which could be used include various insect cell lines such as mosquito larvae (ATCC cell lines CCL 125, 126, 1660, 1591, 6585, 6586), silkworm (ATTC CRL 8851),
10 armyworm (ATCC CRL 1711), moth (ATCC CCL 80) and Drosophila cell lines such as a Schneider cell line (see Schneider J. Embryol. Exp. Morphol. 27:353-365 [1927]). That have been transfected with the appropriate human class I MHC allele encoding genes and the human B₂ microglobulin genes.

15 Peripheral blood lymphocytes are conveniently isolated following simple venipuncture or leukapheresis of normal donors or patients and used as the responder cell sources of CTL precursors. In one embodiment, the appropriate antigen-presenting cells are incubated with 10-100 μ M of
20 peptide in serum-free media for 4 hours under appropriate culture conditions. The peptide-loaded antigen-presenting cells are then incubated with the responder cell populations in vitro for 7 to 10 days under optimized culture conditions. Positive CTL activation can be determined by assaying the
25 cultures for the presence of CTLs that kill radiolabeled target cells, both specific peptide-pulsed targets as well as target cells expressing endogenously processed form of the relevant virus or tumor antigen from which the peptide sequence was derived.

30 Specificity and MHC restriction of the CTL is determined by testing against different peptide target cells expressing appropriate or inappropriate human MHC class I. The peptides that test positive in the MHC binding assays and give rise to specific CTL responses are referred to herein as
35 immunogenic peptides.

The immunogenic peptides can be prepared synthetically, or by recombinant DNA technology or isolated from natural sources such as whole viruses or tumors.

Although the peptide will preferably be substantially free of other naturally occurring host cell proteins and fragments thereof, in some embodiments the peptides can be synthetically conjugated to native fragments or particles. The polypeptides
5 or peptides can be a variety of lengths, either in their neutral (uncharged) forms or in forms which are salts, and either free of modifications such as glycosylation, side chain oxidation, or phosphorylation or containing these
10 modifications, subject to the condition that the modification not destroy the biological activity of the polypeptides as herein described.

Desirably, the peptide will be as small as possible while still maintaining substantially all of the biological activity of the large peptide. When possible, it may be
15 desirable to optimize peptides of the invention to a length of 9 or 10 amino acid residues, commensurate in size with endogenously processed viral peptides or tumor cell peptides that are bound to MHC class I molecules on the cell surface.

Peptides having the desired activity may be modified
20 as necessary to provide certain desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide to bind the desired MHC molecule and activate the appropriate T cell. For instance,
25 the peptides may be subject to various changes, such as substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use, such as improved MHC binding. By conservative substitutions is meant replacing an amino acid residue with
30 another which is biologically and/or chemically similar, e.g., one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as Gly, Ala; Val, Ile, Leu, Met; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. The effect of single amino acid
35 substitutions may also be probed using D-amino acids. Such modifications may be made using well known peptide synthesis procedures, as described in e.g., Merrifield, Science 232:341-347 (1986), Barany and Merrifield, The Peptides, Gross and

Meienhofer, eds. (N.Y., Academic Press), pp. 1-284 (1979); and Stewart and Young, Solid Phase peptide Synthesis, (Rockford, Ill., Pierce), 2d Ed. (1984), incorporated by reference herein.

5 The peptides can also be modified by extending or decreasing the compound's amino acid sequence, e.g., by the addition or deletion of amino acids. The peptides or analogs of the invention can also be modified by altering the order or composition of certain residues, it being readily appreciated
10 that certain amino acid residues essential for biological activity, e.g., those at critical contact sites or conserved residues, may generally not be altered without an adverse effect on biological activity. The non-critical amino acids need not be limited to those naturally occurring in proteins,
15 such as L- α -amino acids, or their D-isomers, but may include non-natural amino acids as well, such as β - γ - δ -amino acids, as well as many derivatives of L- α -amino acids.

 Typically, a series of peptides with single amino acid substitutions are employed to determine the effect of
20 electrostatic charge, hydrophobicity, etc. on binding. For instance, a series of positively charged (e.g., Lys or Arg) or negatively charged (e.g., Glu) amino acid substitutions are made along the length of the peptide revealing different patterns of sensitivity towards various MHC molecules and T
25 cell receptors. In addition, multiple substitutions using small, relatively neutral moieties such as Ala, Gly, Pro, or similar residues may be employed. The substitutions may be homo-oligomers or hetero-oligomers. The number and types of residues which are substituted or added depend on the spacing
30 necessary between essential contact points and certain functional attributes which are sought (e.g., hydrophobicity versus hydrophilicity). Increased binding affinity for an MHC molecule or T cell receptor may also be achieved by such substitutions, compared to the affinity of the parent peptide.
35 In any event, such substitutions should employ amino acid residues or other molecular fragments chosen to avoid, for example, steric and charge interference which might disrupt binding.

Amino acid substitutions are typically of single residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final peptide. Substitutional variants are those in which at least one residue of a peptide has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 4 when it is desired to finely modulate the characteristics of the peptide.

TABLE 4

<u>Original Residue</u>	<u>Exemplary Substitution</u>
Ala	ser
Arg	lys
Asn	gln; his
Asp	glu
Cys	ser
Gln	asn
Glu	asp
Gly	pro
His	asn; gln
Ile	leu; val
Leu	ile; val
Lys	arg
Met	leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

Substantial changes in function (e.g., affinity for MHC molecules or T cell receptors) are made by selecting substitutions that are less conservative than those in Table 4, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in peptide properties will be those in which (a) hydrophilic residue, e.g. seryl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (c) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine.

The peptides may also comprise isosteres of two or more residues in the immunogenic peptide. An isostere as defined here is a sequence of two or more residues that can be substituted for a second sequence because the steric conformation of the first sequence fits a binding site specific for the second sequence. The term specifically includes peptide backbone modifications well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the α -carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. See, generally, Spatola, Chemistry and Biochemistry of Amino Acids, peptides and Proteins, Vol. VII (Weinstein ed., 1983).

Modifications of peptides with various amino acid mimetics or unnatural amino acids are particularly useful in increasing the stability of the peptide in vivo. Stability can be assayed in a number of ways. For instance, peptidases and various biological media, such as human plasma and serum, have been used to test stability. See, e.g., Verhoef et al.,

Eur. J. Drug Metab Pharmacokin. 11:291-302 (1986). Half life of the peptides of the present invention is conveniently determined using a 25% human serum (v/v) assay. The protocol is generally as follows. Pooled human serum (Type AB, non-heat inactivated) is delipidated by centrifugation before use. The serum is then diluted to 25% with RPMI tissue culture media and used to test peptide stability. At predetermined time intervals a small amount of reaction solution is removed and added to either 6% aqueous trichloroacetic acid or ethanol. The cloudy reaction sample is cooled (4°C) for 15 minutes and then spun to pellet the precipitated serum proteins. The presence of the peptides is then determined by reversed-phase HPLC using stability-specific chromatography conditions.

The peptides of the present invention or analogs thereof which have CTL stimulating activity may be modified to provide desired attributes other than improved serum half life. For instance, the ability of the peptides to induce CTL activity can be enhanced by linkage to a sequence which contains at least one epitope that is capable of inducing a T helper cell response. Particularly preferred immunogenic peptides/T helper conjugates are linked by a spacer molecule. The spacer is typically comprised of relatively small, neutral molecules, such as amino acids or amino acid mimetics, which are substantially uncharged under physiological conditions. The spacers are typically selected from, e.g., Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. It will be understood that the optionally present spacer need not be comprised of the same residues and thus may be a hetero- or homo-oligomer. When present, the spacer will usually be at least one or two residues, more usually three to six residues. Alternatively, the CTL peptide may be linked to the T helper peptide without a spacer.

The immunogenic peptide may be linked to the T helper peptide either directly or via a spacer either at the amino or carboxy terminus of the CTL peptide. The amino terminus of either the immunogenic peptide or the T helper peptide may be acylated.

In some embodiments it may be desirable to include in the pharmaceutical compositions of the invention at least one component which assists in priming CTL. Lipids have been identified as agents capable of assisting the priming CTL in vivo against viral antigens. For example, palmitic acid residues can be attached to the alpha and epsilon amino groups of a Lys residue and then linked, e.g., via one or more linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to an immunogenic peptide. The lipidated peptide can then be injected directly in a micellar form, incorporated into a liposome or emulsified in an adjuvant, e.g., incomplete Freund's adjuvant. In a preferred embodiment a particularly effective immunogen comprises palmitic acid attached to alpha and epsilon amino groups of Lys, which is attached via linkage, e.g., Ser-Ser, to the amino terminus of the immunogenic peptide.

As another example of lipid priming of CTL responses, E. coli lipoproteins, such as tripalmitoyl-S-glycerylcysteinylserine (P_3CSS) can be used to prime virus specific CTL when covalently attached to an appropriate peptide. See, Deres et al., Nature 342:561-564 (1989), incorporated herein by reference. Peptides of the invention can be coupled to P_3CSS , for example, and the lipopeptide administered to an individual to specifically prime a CTL response to the target antigen. Further, as the induction of neutralizing antibodies can also be primed with P_3CSS conjugated to a peptide which displays an appropriate epitope, the two compositions can be combined to more effectively elicit both humoral and cell-mediated responses to infection.

In addition, additional amino acids can be added to the termini of a peptide to provide for ease of linking peptides one to another, for coupling to a carrier support, or larger peptide, for modifying the physical or chemical properties of the peptide or oligopeptide, or the like. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide or oligopeptide. Modification at the C

terminus in some cases may alter binding characteristics of the peptide. In addition, the peptide or oligopeptide sequences can differ from the natural sequence by being modified by terminal-NH₂ acylation, e.g., by alkanoyl (C₁-C₂₀) or thioglycolyl acetylation, terminal-carboxyl amidation, 5 e.g., ammonia, methylamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

The peptides of the invention can be prepared in a wide variety of ways. Because of their relatively short size, 10 the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, 15 Stewart and Young, Solid Phase Peptide Synthesis, 2d. ed., Pierce Chemical Co. (1984), *supra*.

Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes an immunogenic peptide of interest is inserted into an expression 20 vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York 25 (1982), which is incorporated herein by reference. Thus, fusion proteins which comprise one or more peptide sequences of the invention can be used to present the appropriate T cell epitope.

As the coding sequence for peptides of the length 30 contemplated herein can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al., J. Am. Chem. Soc. 103:3185 (1981), modification can be made simply by substituting the appropriate base(s) for those encoding the native peptide sequence. The coding sequence can 35 then be provided with appropriate linkers and ligated into expression vectors commonly available in the art, and the vectors used to transform suitable hosts to produce the desired fusion protein. A number of such vectors and suitable

host systems are now available. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions and usually a replication system to provide an expression
5 vector for expression in the desired cellular host. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable
10 bacterial hosts. Of course, yeast or mammalian cell hosts may also be used, employing suitable vectors and control sequences.

The peptides of the present invention and pharmaceutical and vaccine compositions thereof are useful for
15 administration to mammals, particularly humans, to treat and/or prevent viral infection and cancer. Examples of diseases which can be treated using the immunogenic peptides of the invention include prostate cancer, hepatitis B, hepatitis C, AIDS, renal carcinoma, cervical carcinoma,
20 lymphoma, CMV and condyloma acuminatum.

For pharmaceutical compositions, the immunogenic peptides of the invention are administered to an individual already suffering from cancer or infected with the virus of interest. Those in the incubation phase or the acute phase of
25 infection can be treated with the immunogenic peptides separately or in conjunction with other treatments, as appropriate. In therapeutic applications, compositions are administered to a patient in an amount sufficient to elicit an effective CTL response to the virus or tumor antigen and to
30 cure or at least partially arrest symptoms and/or complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, e.g., the peptide composition, the manner of administration, the stage and
35 severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician, but generally range for the initial immunization (that is for therapeutic or prophylactic

administration) from about 1.0 μ g to about 5000 μ g of peptide for a 70 kg patient, followed by boosting dosages of from about 1.0 μ g to about 1000 μ g of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific CTL activity in the patient's blood. It must be kept in mind that the peptides and compositions of the present invention may generally be employed in serious disease states; that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of the peptides, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these peptide compositions.

For therapeutic use, administration should begin at the first sign of viral infection or the detection or surgical removal of tumors or shortly after diagnosis in the case of acute infection. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. In chronic infection, loading doses followed by boosting doses may be required.

Treatment of an infected individual with the compositions of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals susceptible (or predisposed) to developing chronic infection the compositions are particularly useful in methods for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or during infection, for instance, as described herein, the composition can be targeted to them, minimizing need for administration to a larger population.

The peptide compositions can also be used for the treatment of chronic infection and to stimulate the immune system to eliminate virus-infected cells in carriers. It is important to provide an amount of immuno-potentiating peptide in a formulation and mode of administration sufficient to effectively stimulate a cytotoxic T cell response. Thus, for treatment of chronic infection, a representative dose is in

the range of about 1.0 μg to about 5000 μg , preferably about 5 μg to 1000 μg for a 70 kg patient per dose. Immunizing doses followed by boosting doses at established intervals, e.g., from one to four weeks, may be required, possibly for a
5 prolonged period of time to effectively immunize an individual. In the case of chronic infection, administration should continue until at least clinical symptoms or laboratory tests indicate that the viral infection has been eliminated or substantially abated and for a period thereafter.

10 The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral or local administration. Preferably, the pharmaceutical compositions are administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the
15 invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.9% saline, 0.3% glycine,
20 hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution
25 prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium
30 lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of CTL stimulatory peptides of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about
35 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

The peptides of the invention may also be administered via liposomes, which serve to target the peptides to a particular tissue, such as lymphoid tissue, or targeted selectively to infected cells, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, e.g., a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes filled with a desired peptide of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, incorporated herein by reference.

For targeting to the immune cells, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a peptide may be administered intravenously, locally, topically, etc. in a dose which varies according to, inter alia, the manner of administration, the peptide being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose,

sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the immunogenic peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of peptides are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

In another aspect the present invention is directed to vaccines which contain as an active ingredient an immunogenically effective amount of an immunogenic peptide as described herein. The peptide(s) may be introduced into a host, including humans, linked to its own carrier or as a homopolymer or heteropolymer of active peptide units. Such a polymer has the advantage of increased immunological reaction and, where different peptides are used to make up the polymer, the additional ability to induce antibodies and/or CTLs that react with different antigenic determinants of the virus or tumor cells. Useful carriers are well known in the art, and include, e.g., thyroglobulin, albumins such as bovine serum albumin, tetanus toxoid, polyamino acids such as poly(lysine:glutamic acid), hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like. The

vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, or saline, and further typically include an adjuvant.

5 Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are materials well known in the art. And, as mentioned above, CTL responses can be primed by conjugating peptides of the invention to lipids, such as P₃CSS. Upon immunization with a peptide composition as described herein, via injection, aerosol, oral, transdermal
10 or other route, the immune system of the host responds to the vaccine by producing large amounts of CTLs specific for the desired antigen, and the host becomes at least partially immune to later infection, or resistant to developing chronic infection.

15 Vaccine compositions containing the peptides of the invention are administered to a patient susceptible to or otherwise at risk of viral infection or cancer to elicit an immune response against the antigen and thus enhance the patient's own immune response capabilities. Such an amount is
20 defined to be an "immunogenically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc., but generally range from about 1.0 µg to about 5000 µg per 70 kilogram patient, more commonly
25 from about 10 µg to about 500 µg mg per 70 kg of body weight.

In some instances it may be desirable to combine the peptide vaccines of the invention with vaccines which induce neutralizing antibody responses to the virus of interest, particularly to viral envelope antigens.

30 For therapeutic or immunization purposes, the peptides of the invention can also be expressed by attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus as a vector to express nucleotide sequences that encode the peptides of the
35 invention. Upon introduction into an acutely or chronically infected host or into a non-infected host, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits a host CTL response. Vaccinia vectors and methods

useful in immunization protocols are described in, e.g., U.S. Patent No. 4,722,848, incorporated herein by reference. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover et al. (Nature 351:456-460 (1991)) which is incorporated herein by reference. A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g., Salmonella typhi vectors and the like, will be apparent to those skilled in the art from the description herein.

Antigenic peptides may be used to elicit CTL ex vivo, as well. The resulting CTL, can be used to treat chronic infections (viral or bacterial) or tumors in patients that do not respond to other conventional forms of therapy, or will not respond to a peptide vaccine approach of therapy. Ex vivo CTL responses to a particular pathogen (infectious agent or tumor antigen) are induced by incubating in tissue culture the patient's CTL precursor cells (CTLp) together with a source of antigen-presenting cells (APC) and the appropriate immunogenic peptide. After an appropriate incubation time (typically 1-4 weeks), in which the CTLp are activated and mature and expand into effector CTL, the cells are infused back into the patient, where they will destroy their specific target cell (an infected cell or a tumor cell). In order to optimize the *in vitro* conditions for the generation of specific cytotoxic T cells, the culture of stimulator cells is maintained in an appropriate serum-free medium.

Prior to incubation of the stimulator cells with the cells to be activated, e.g., precursor CD8+ cells, an amount of antigenic peptide is added to the stimulator cell culture, of sufficient quantity to become loaded onto the human Class I molecules to be expressed on the surface of the stimulator cells. In the present invention, a sufficient amount of peptide is an amount that will allow about 200, and preferably 200 or more, human Class I MHC molecules loaded with peptide to be expressed on the surface of each stimulator cell. Preferably, the stimulator cells are incubated with >20µg/ml peptide.

Resting or precursor CD8+ cells are then incubated in culture with the appropriate stimulator cells for a time period sufficient to activate the CD8+ cells. Preferably, the CD8+ cells are activated in an antigen-specific manner. The ratio of resting or precursor CD8+ (effector) cells to stimulator cells may vary from individual to individual and may further depend upon variables such as the amenability of an individual's lymphocytes to culturing conditions and the nature and severity of the disease condition or other condition for which the within-described treatment modality is used. Preferably, however, the lymphocyte:stimulator cell ratio is in the range of about 30:1 to 300:1. The effector/stimulator culture may be maintained for as long a time as is necessary to stimulate a therapeutically useable or effective number of CD8+ cells.

The induction of CTL *in vitro* requires the specific recognition of peptides that are bound to allele specific MHC class I molecules on APC. The number of specific MHC/peptide complexes per APC is crucial for the stimulation of CTL, particularly in primary immune responses. While small amounts of peptide/MHC complexes per cell are sufficient to render a cell susceptible to lysis by CTL, or to stimulate a secondary CTL response, the successful activation of a CTL precursor (pCTL) during primary response requires a significantly higher number of MHC/peptide complexes. Peptide loading of empty major histocompatibility complex molecules on cells allows the induction of primary cytotoxic T lymphocyte responses. Peptide loading of empty major histocompatibility complex molecules on cells enables the induction of primary cytotoxic T lymphocyte responses.

Since mutant cell lines do not exist for every human MHC allele, it is advantageous to use a technique to remove endogenous MHC-associated peptides from the surface of APC, followed by loading the resulting empty MHC molecules with the immunogenic peptides of interest. The use of non-transformed (non-tumorigenic), non-infected cells, and preferably, autologous cells of patients as APC is desirable for the design of CTL induction protocols directed towards development

of ex vivo CTL therapies. This application discloses methods for stripping the endogenous MHC-associated peptides from the surface of APC followed by the loading of desired peptides.

A stable MHC class I molecule is a trimeric complex formed of the following elements: 1) a peptide usually of 8 - 10 residues, 2) a transmembrane heavy polymorphic protein chain which bears the peptide-binding site in its $\alpha 1$ and $\alpha 2$ domains, and 3) a non-covalently associated non-polymorphic light chain, β_2 microglobulin. Removing the bound peptides and/or dissociating the β_2 microglobulin from the complex renders the MHC class I molecules nonfunctional and unstable, resulting in rapid degradation. All MHC class I molecules isolated from PBMCs have endogenous peptides bound to them. Therefore, the first step is to remove all endogenous peptides bound to MHC class I molecules on the APC without causing their degradation before exogenous peptides can be added to them.

Two possible ways to free up MHC class I molecules of bound peptides include lowering the culture temperature from 37°C to 26°C overnight to destabilize β_2 microglobulin and stripping the endogenous peptides from the cell using a mild acid treatment. The methods release previously bound peptides into the extracellular environment allowing new exogenous peptides to bind to the empty class I molecules. The cold-temperature incubation method enables exogenous peptides to bind efficiently to the MHC complex, but requires an overnight incubation at 26°C which may slow the cell's metabolic rate. It is also likely that cells not actively synthesizing MHC molecules (e.g., resting PBMC) would not produce high amounts of empty surface MHC molecules by the cold temperature procedure.

Harsh acid stripping involves extraction of the peptides with trifluoroacetic acid, pH 2, or acid denaturation of the immunoaffinity purified class I-peptide complexes. These methods are not feasible for CTL induction, since it is important to remove the endogenous peptides while preserving APC viability and an optimal metabolic state which is critical for antigen presentation. Mild acid solutions of pH 3 such as

glycine or citrate-phosphate buffers have been used to identify endogenous peptides and to identify tumor associated T cell epitopes. The treatment is especially effective, in that only the MHC class I molecules are destabilized (and associated peptides released), while other surface antigens remain intact, including MHC class II molecules. Most importantly, treatment of cells with the mild acid solutions do not affect the cell's viability or metabolic state. The mild acid treatment is rapid since the stripping of the endogenous peptides occurs in two minutes at 4°C and the APC is ready to perform its function after the appropriate peptides are loaded. The technique is utilized herein to make peptide-specific APCs for the generation of primary antigen-specific CTL. The resulting APC are efficient in inducing peptide-specific CD8+ CTL.

Activated CD8+ cells may be effectively separated from the stimulator cells using one of a variety of known methods. For example, monoclonal antibodies specific for the stimulator cells, for the peptides loaded onto the stimulator cells, or for the CD8+ cells (or a segment thereof) may be utilized to bind their appropriate complementary ligand. Antibody-tagged molecules may then be extracted from the stimulator-effector cell admixture via appropriate means, e.g., via well-known immunoprecipitation or immunoassay methods.

Effective, cytotoxic amounts of the activated CD8+ cells can vary between *in vitro* and *in vivo* uses, as well as with the amount and type of cells that are the ultimate target of these killer cells. The amount will also vary depending on the condition of the patient and should be determined via consideration of all appropriate factors by the practitioner. Preferably, however, about 1×10^6 to about 1×10^{12} , more preferably about 1×10^8 to about 1×10^{11} , and even more preferably, about 1×10^9 to about 1×10^{10} activated CD8+ cells are utilized for adult humans, compared to about 5×10^6 - 5×10^7 cells used in mice.

Preferably, as discussed above, the activated CD8+ cells are harvested from the cell culture prior to

administration of the CD8+ cells to the individual being treated. It is important to note, however, that unlike other present and proposed treatment modalities, the present method uses a cell culture system that is not tumorigenic.

5 Therefore, if complete separation of stimulator cells and activated CD8+ cells is not achieved, there is no inherent danger known to be associated with the administration of a small number of stimulator cells, whereas administration of mammalian tumor-promoting cells may be extremely hazardous.

10 Methods of re-introducing cellular components are known in the art and include procedures such as those exemplified in U.S. Patent No. 4,844,893 to Honsik, et al. and U.S. Patent No. 4,690,915 to Rosenberg. For example, administration of activated CD8+ cells via intravenous
15 infusion is appropriate.

The immunogenic peptides of this invention may also be used to make monoclonal antibodies. Such antibodies may be useful as potential diagnostic or therapeutic agents.

The peptides may also find use as diagnostic
20 reagents. For example, a peptide of the invention may be used to determine the susceptibility of a particular individual to a treatment regimen which employs the peptide or related peptides, and thus may be helpful in modifying an existing treatment protocol or in determining a prognosis for an
25 affected individual. In addition, the peptides may also be used to predict which individuals will be at substantial risk for developing chronic infection.

The following examples are offered by way of illustration, not by way of limitation.

30

Example 1

Class I antigen isolation

A flow diagram of an HLA-A antigen purification scheme is presented in Figure 1. Briefly, the cells bearing
35 the appropriate allele were grown in large batches (6-8 liters yielding $\sim 5 \times 10^9$ cells), harvested by centrifugation and washed. All cell lines were maintained in RPMI 1640 media (Sigma) supplemented with 10% fetal bovine serum (FBS) and

antibiotics. For large-scale cultures, cells were grown in roller bottle culture in RPMI 1640 with 10% FBS or with 10% horse serum and antibiotics. Cells were harvested by centrifugation at 1500 RPM IEC-CRU5000 centrifuge with 259 rotor and washed three times with phosphate-buffered saline (PBS) (0.01 M PO_4 , 0.154 M NaCl, pH 7.2).

Cells were pelleted and stored at -70°C or treated with detergent lysing solution to prepare detergent lysates. Cell lysates were prepared by the addition of stock detergent solution [1% NP-40 (Sigma) or Renex 30 (Accurate Chem. Sci. Corp., Westbury, NY 11590), 150 mM NaCl, 50 mM Tris, pH 8.0] to the cell pellets (previously counted) at a ratio of 50-100 $\times 10^6$ cells per ml detergent solution. A cocktail of protease inhibitors was added to the premeasured volume of stock detergent solution immediately prior to the addition to the cell pellet. Addition of the protease inhibitor cocktail produced final concentrations of the following: phenylmethylsulfonyl fluoride (PMSF), 2 mM; aprotinin, 5 $\mu\text{g/ml}$; leupeptin, 10 $\mu\text{g/ml}$; pepstatin, 10 $\mu\text{g/ml}$; iodoacetamide, 100 μM ; and EDTA, 3 ng/ml. Cell lysis was allowed to proceed at 4°C for 1 hour with periodic mixing. Routinely $5-10 \times 10^9$ cells were lysed in 50-100 ml of detergent solution. The lysate was clarified by centrifugation at $15,000 \times g$ for 30 minutes at 4°C and subsequent passage of the supernatant fraction through a 0.2μ filter unit (Nalgene).

The HLA-A antigen purification was achieved using affinity columns prepared with mAb-conjugated Sepharose beads. For antibody production, cells were grown in RPMI with 10% FBS in large tissue culture flasks (Corning 25160-225). Antibodies were purified from clarified tissue culture medium by ammonium sulfate fractionation followed by affinity chromatography on protein-A-Sepharose (Sigma). Briefly, saturated ammonium sulfate was added slowly with stirring to the tissue culture supernatant to 45% (volume to volume) overnight at 4°C to precipitate the immunoglobulins. The precipitated proteins were harvested by centrifugation at $10,000 \times g$ for 30 minutes. The precipitate was then dissolved

in a minimum volume of PBS and transferred to dialysis tubing (Spectro/Por 2, Mol. wt. cutoff 12,000-14,000, Spectrum Medical Ind.). Dialysis was against PBS (≥ 20 times the protein solution volume) with 4-6 changes of dialysis buffer over a
5 24-48 hour period at 4°C. The dialyzed protein solution was clarified by centrifugation (10,000 x g for 30 minutes) and the pH of the solution adjusted to pH 8.0 with 1N NaOH. Protein-A-Sepharose (Sigma) was hydrated according to the manufacturer's instructions, and a protein-A-Sepharose column
10 was prepared. A column of 10 ml bed volume typically binds 50-100 mg of mouse IgG.

The protein sample was loaded onto the protein-A-Sepharose column using a peristaltic pump for large loading volumes or by gravity for smaller volumes (<100 ml). The
15 column was washed with several volumes of PBS, and the eluate was monitored at A280 in a spectrophotometer until base line was reached. The bound antibody was eluted using 0.1 M citric acid at suitable pH (adjusted to the appropriate pH with 1N NaOH). For mouse IgG-1 pH 6.5 was used for IgG2a pH 4.5 was
20 used and for IgG2b and IgG3 pH 3.0 was used. 2 M Tris base was used to neutralize the eluate. Fractions containing the antibody (monitored by A280) were pooled, dialyzed against PBS and further concentrated using an Amicon Stirred Cell system (Amicon Model 8050 with YM30 membrane). The anti-A2 mAb,
25 BB7.2, and the anti-A3 mAb, GAPA3, are particularly useful for affinity purification.

The HLA-A antigen was purified using affinity columns prepared with mAb-conjugated Sepharose beads. The affinity columns were prepared by incubating protein-A-Sepharose beads
30 (Sigma) with affinity-purified mAb as described above. Five to 10 mg of mAb per ml of bead is the preferred ratio. The mAb bound beads were washed with borate buffer (borate buffer: 100 mM sodium tetraborate, 154 mM NaCl, pH 8.2) until the washes show A280 at based line. Dimethyl pimelimidate (20 mM)
35 in 200 mM triethanolamine was added to covalently crosslink the bound mAb to the protein-A-Sepharose (Schneider et al., J. Biol. Chem. 257:10766 (1982)). After incubation for 45 minutes at room temperature on a rotator, the excess crosslinking

reagent was removed by washing the beads twice with 10-20 ml of 20 mM ethanolamine, pH 8.2. Between each wash the slurry was placed on a rotator for 5 minutes at room temperature. The beads were washed with borate buffer and with PBS plus 0.02% sodium azide.

The cell lysate ($5-10 \times 10^9$ cell equivalents) was then slowly passed over a 5-10 ml affinity column (flow rate of 0.1-0.25 ml per minute) to allow the binding of the antigen to the immobilized antibody. After the lysate was allowed to pass through the column, the column was washed sequentially with 20 column volumes of detergent stock solution plus 0.1% sodium dodecyl sulfate, 20 column volumes of 0.5 M NaCl, 20 mM Tris, pH 8.0, and 10 column volumes of 20 mM Tris, pH 8.0. The HLA-A antigen bound to the mAb was eluted with a basic buffer solution (50 mM diethylamine in water). As an alternative, acid solutions such as 0.15-0.25 M acetic acid were also used to elute the bound antigen. An aliquot of the eluate (1/50) was removed for protein quantification using either a colorimetric assay (BCA assay, Pierce) or by SDS-PAGE, or both. SDS-PAGE analysis was performed as described by Laemmli (Laemmli, U.K., *Nature* 227:680 (1970)) using known amounts of bovine serum albumin (Sigma) as a protein standard.

Allele specific antibodies were used to purify the specific MHC molecule. In the case of HLA-A2 and HLA-A3 mAbs BB7.2 and GAPA3 were used respectively. An example of SDS PAGE analysis of purified HLA-A3.2 molecules is shown in Figure 2.

Figure 2 shows SDS-PAGE (12.5%) analysis of affinity purified HLA-A3.2 from the cell line EHM. An affinity column (10 ml) was prepared with protein A-sepharose beads coupled to the monoclonal antibody GAPA3 which is specific for HLA-A3. A detergent lysate of 5×10^9 cells was passaged over the column and the column was washed extensively. The bound HLA-A3.2 molecules were eluted from the column with 0.15M acetic acid, 50 ml. One ml of the eluate was removed and lyophilized to concentrate the sample. The sample was taken up to 50 μ l with Laemmli sample buffer and 20 μ l were loaded in lane 2. Lane 1 contained molecular weight standards: Myosin, 230 kD; B-

galactosidase, 116kD; phosphorylase B, 97.4kD; bovine serum albumin, 66.2kD; ovalbumin, 45kD; carbonic anhydrase, 31kD; soybean trypsin inhibitor, 21.5kD; and lysozyme, 14.4kD.

Standard concentrations of bovine serum albumin were run in lanes 8, 10 μ g, 9, 3 μ g, and 10, 1 μ g to aid in the estimation of protein yield. For this particular HLA-A3.2 preparation, the estimated yield was approximately 112 μ g.

For HLA-A11, A24.1 and A1, an alternative protocol was used whereby anti-HLA-B and C monoclonal antibodies were used to deplete HLA-B and C molecules. The remaining HLA-A molecules were subsequently purified using the W6/32 mAb as described below.

Based on the density of class I expression as indicated by the results of immunofluorescent staining analysis, it is anticipated that average yields of class I antigen isolated from the EBV B cell lines will range from 800-1200 μ g per 10^{10} cell equivalents.

Example 2

An alternative class I purification protocol

HLA-A2.1 molecules were isolated using the mAb B1.23.2 which detects an epitope expressed by HLA-B and C allele molecules, but not by HLA-A antigens. The mAb, W6/32, detects all human class I molecules, including HLA-A, B and C. As mentioned above, these mAbs react well with the B cell lines serving as sources of HLA-A antigens. The B1.23.2 mAb reacts with the various human B cell lines, but fails to react with a mouse cell line that expresses a transfected HLA-A2.1 protein or a chimeric A2.1 mouse K^b molecule. It does react with the human cell line, CIR (Alexander, J., et al., Immunogenetics, 29, 380 [1989]), that lacks expression of HLA-A and B molecules, but expresses low levels of HLA-C molecules. This pattern of reactivity illustrates how the B1.23.2 mAb can be used to deplete the B cell lysates of HLA-B and C molecules.

Affinity columns were prepared using the affinity-purified B1.23.2 and W6/32 mAbs, respectively, as described above. The procedures for the preparation of the affinity

columns are essentially identical to the procedures described for the preparation of the allele-specific mAb columns described above. The B1.23.2 mAb affinity column was used to deplete the detergent lysates of HLA-B and C molecules using the protocol as described above. The cell lysate depleted of HLA-B and C was then passed over a W6/32 mAb affinity column. The MHC molecule that was eluted from this second passage was the A allele product.

This alternative affinity purification is useful for the purification of any HLA-A allele product, and does not rely on the need for allele-specific mAbs. In addition, it could also be used to isolate any class I molecule type from transfected cell lines.

15

Example 3

Isolation and sequencing of naturally processed peptides

For the HLA-A preparations derived from the base (50 mM diethylamine) elution protocol, the eluate was immediately neutralized with 1 N acetic acid to pH 7.0-7.5. The neutralized eluate was concentrated to a volume of 1-2 ml in an Amicon stirred cell [Model 8050, with YM3 membranes (Amicon)]. Ten ml of ammonium acetate (0.01 M, pH 8.0) was added to the concentrator to remove the non-volatile salts, and the sample was concentrated to approximately 1 ml. A small sample (1/50) was removed for protein quantitation as described above. The remainder was recovered into a 15 ml polypropylene conical centrifuge tube (Falcon, 2097) (Becton Dickinson). Glacial acetic acid was added to obtain a final concentration of 10% acetic acid. The acidified sample was placed in a boiling water bath for 5 minutes to allow for the dissociation of the bound peptides. The sample was cooled on ice, returned to the concentrator and the filtrate was collected. Additional aliquots of 10% acetic acid (1-2 ml) were added to the concentrator, and this filtrate was pooled with the original filtrate. Finally, 1-2 ml of distilled water was added to the concentrator, and this filtrate was pooled as well.

The retentate contains the bulk of the HLA-A heavy chain and β_2 -microglobulin, while the filtrate contains the naturally processed bound peptides and other components with molecular weights less than about 3000. The pooled filtrate material was lyophilized in order to concentrate the peptide fraction. The sample was then ready for further analysis.

For HPLC (high performance liquid chromatography) separation of the peptide fractions, the lyophilized sample was dissolved in 50 μ l of distilled water, or into 0.1% trifluoroacetic acid (TFA) (Applied Biosystems) in water and injected to a C18 reverse-phase narrow bore column (Beckman C18 Ultrasphere, 10 x 250 mm), using a gradient system described by Stone and Williams (Stone, K.L. and Williams K.R., in, *Macromolecular Sequencing and Synthesis; Selected Methods and Applications*, A.R. Liss, New York, 1988, pp. 7-24. Buffer A was 0.06% TFA in water (Burdick-Jackson) and buffer B was 0.052% TFA in 80% acetonitrile (Burdick-Jackson). The flow rate was 0.250 ml/minute with the following gradient: 0-60 min., 2-37.5% B; 60-95 min., 37.5-75% B; 95-105 min., 75-98% B. The Gilson narrow bore HPLC configuration is particularly useful for this purpose, although other configurations work equally well.

A large number of peaks were detected by absorbance at 214 nm, many of which appear to be of low abundance (Fig. 3). Whether a given peak represents a single peptide or a peptide mixture was not determined. Pooled fractions were then sequenced to determine motifs specific for each allele as described below.

Pooled peptide fractions, prepared as described above were analyzed by automated Edman sequencing using the Applied Biosystems Model 477A automated sequencer. The sequencing method is based on the technique developed by Pehr Edman in the 1950s for the sequential degradation of proteins and peptides to determine the sequence of the constituent amino acids.

The protein or peptide to be sequenced was held by a 12-mm diameter porous glass fiber filter disk in a heated, argon-purged reaction chamber. The filter was generally pre-

treated with BioBrene PlusTM and then cycled through one or more repetitions of the Edman reaction to reduce contaminants and improve the efficiency of subsequent sample sequencing. Following the pre-treatment of the filter, a solution of the sample protein or peptide (10 pmol-5 nmol range) was loaded onto the glass filter and dried. Thus, the sample was left embedded in the film of the pre-treated disk. Covalent attachment of the sample to the filter was usually not necessary because the Edman chemistry utilized relatively apolar solvents, in which proteins and peptides are poorly soluble.

Briefly, the Edman degradation reaction has three steps: coupling, cleavage, and conversion. In coupling step, phenylisothiocyanate (PITC) is added. The PITC reacts quantitatively with the free amino-terminal amino acid of the protein to form the phenylthiocarbamyl-protein in a basic environment. After a period of time for the coupling step, the excess chemicals are extracted and the highly volatile organic acid, trifluoroacetic acid, TFA, is used to cleave the PITC-coupled amino acid residue from the amino terminus of the protein yielding the anilinothiazolinone (ATZ) derivative of the amino acid. The remaining protein/peptide is left with a new amino terminus and is ready for the next Edman cycle. The ATZ amino acid is extracted and transferred to a conversion flask, where upon addition of 25% TFA in water, the ATZ amino acid is converted to the more stable phenylthiohydantoin (PTH) amino acid that can be identified and quantified following automatic injection into the Model 120 PTH Analyzer which uses a microbore C-18 reverse-phase HPLC column for the analysis.

In the present procedures, peptide mixtures were loaded onto the glass filters. Thus, a single amino acid sequence usually does not result. Rather, mixtures of amino acids in different yield are found. When the particular residue is conserved among the peptides being sequenced, increased yield for that amino acid is observed.

Example 4

Definition of an A3.2 specific motif

There is some ambiguity in the international nomenclature of A3 alleles. The A3.2 allele herein is expressed by cell lines EHM, HO301, and GM3107. This particular subtype is currently referred to as the 3.2 allele (Yang, in Immunobiology of HLA, Vol. 1, Dupont ed., Springer-Verlag, New York pp. 43-44 and 54-55, 1989), or the product of the A*0301 gene (its sequence corresponds to the one published by Strachan, et al., EMBO J., 3:887 (1984), and has been verified by direct cloning and sequencing of the A3 gene found in EHM cell line. The HLA-A3.2 encoded by the A*0301 gene referred to in this document is the commonly expressed HLA-A3 allelic form.

In one case using MAT cells, pooled peptide fractions prepared as described in Example 3 above were obtained from HLA-A3.2 homozygous cell lines, for example, CM3107. The pooled fractions were HPLC fractions corresponding to 7% to 19% CH_3CN . For this class I molecule, this region of the chromatogram was most abundant in peptides. Data from independent experiments were averaged as described below.

The amino acid sequence analyses from four independent experiments were analyzed and the results are shown in Table 5. For each position except the first, the data were analyzed by modifying the method described by Falk et al. to allow for comparison of experiments from different HLA types. This modified procedure yielded quantitative yet standardized values while allowing the averaging of data from different experiments involving the same HLA type.

The raw sequenator data was converted to a simple matrix of 10 rows (each representing one Edman degradation cycle) and 16 columns (each representing one of the twenty amino acids; W, C, R and H were eliminated for technical reasons. The data corresponding to the first row (first cycle) was not considered further because, this cycle is usually heavily contaminated by free amino acids.). The values of each row were summed to yield a total pmoles value for that particular cycle. For each row, values for each amino acid were then divided by the corresponding total yield value, to determine what fraction of the total signal is

attributable to each amino acid at each cycle. By doing so, an "Absolute Frequency" table was generated. This absolute frequency table allows correction for the declining yields of each cycle.

5 Starting from the absolute frequency table, a "relative frequency" table was then generated to allow comparisons among different amino acids. To do so the data from each column was summed, and then averaged. Then, each value was divided next by the average column value to obtain
10 relative frequency values. These values quantitate, in a standardized manner, increases and decreases per cycle, for each of the different sixteen amino acid types. Tables generated from data from different experiments can thus be added together to generate average relative frequency values
15 (and their standard deviations). All standard deviations can then be averaged, to estimate a standard deviation value applicable to the samples from each table. Any particular value exceeding 1.00 by more than two standard deviations is considered to correspond to a significant increase.

20 The results of the foregoing analysis for HLA-A3.2 were as follows: at position 2, a 2.2-fold increase in valine (V) with lesser increases (1.5-1.7) for structurally similar residues leucine (L) and methionine (M). At position 3, tyrosine (Y) and aspartic acid (D) showed increases in
25 frequency. At position 7 isoleucine (I) was increased, and at position 8 asparagine (N) and glutamine (Q) were increased. At positions 9 and 10, lysine (K) was increased more than 2-fold over the expected random yield.

 Cysteine was not modified and thus not detected.
30 PTH-tryptophan coeluted with diphenylurea, and in some experiments, PTH-arginine coeluted with the major derivative of PTH-threonine. Therefore, cysteine and tryptophan are not detectable and arginine is detected only in the absence of threonine.

35 Previously described MHC structures showed instances of critically conserved residues at position 2 (or 3) and at the C terminus (either position 9 or 10). These residues are referred to as "conserved" residues. The modified data

analysis of this invention considered the conserved positions at the N and C terminals.

Thus, the HLA-A3.2 motif should have position two occupied by V, L or M, a length of 9 or 10 amino acids, and a C-terminal position occupied by K.

TABLE 5
Summary
HLA-A3.2 Allele-Specific Motif

	Position	Conserved Residues
	1	-
	2	V,L,M
15	3	Y,D
	4	-
	5	-
	6	-
	7	I
20	8	Q,N
	9	K
	10	K

Example 5

Definition of HLA-A1-specific peptide motifs

HLA-A1 molecules were isolated and their naturally processed peptides characterized, as described in Example 3 above. In one case using MAT cells, pooled fractions corresponding to 19% to 50% CH₃CN were used. As in the preceding example, residues showing at any given position except the first position, at least a two standard deviation increase over the random expected yield were identified and shown in Table 6. On the basis of these data, only Serine (S) and Threonine (T) were increased at position two. At position 3, aspartic acid (D) and glutamic acid (E) were elevated and at position 9 and 10 tyrosine (Y) showed a marked increase. Other increases noted were proline (P) at position 4 and leucine (L) at position 7. Therefore, the motifs for HLA-A1 based on these data would have residues at position 2 occupied by S or T, a peptide length of 9 or 10 amino acids and a

C-terminal residue of Y. Alternatively, another motif would comprise a D or E at position 3 together with a C terminal residue of Y.

TABLE 6
Summary
HLA-A1 Allele-Specific Motif

	Position	Conserved Residues
10	1	-
	2	S, T
	3	D,E
	4	P
	5	-
15	6	-
	7	L
	8	-
	9	Y

Example 6

Definition of HLA-A11 allele-specific peptide motifs

HLA-A11 motifs were defined by amino acid sequence analysis of pooled HPLC fractions, in one case corresponding to 7% to 45% CH₃CN of fractionated peptides eluted from HLA-A11 molecules purified from the cell line BVR. On the basis of the data presented in Table 7, a motif for A11 consists of a conserved residue at position 2 of threonine (T) or valine (V), a peptide length of 9 or 10 amino acids, and a C-terminal conserved residue of lysine (K). At position 3 increases in methionine (M) and phenylalanine (F) were also seen and at position 8 glutamine (Q) was increased.

TABLE 7
Summary
HLA-A11 Allele-Specific Motif

	Position	Conserved Residues
	1	-
40	2	T,V
	3	M,F
	4	-

47

	5	-
	6	-
	7	-
	8	Q
5	9	K
	10	K

Example 7Definition of HLA-A24.1 Specific Peptide Motifs

10 HLA-A24.1 allele-specific motifs were defined by amino acid sequence analysis of pooled fractions in one case corresponding to 7% to 19% CH₃CN of HPLC fractionated peptides eluted from HLA-A24.1 molecules purified from the cell line KT3. On the basis of the data presented in Table 8 a motif

15 for HLA-A24.1 consists of a conserved residue at position 2 occupied by tyrosine (Y), a peptide length of 9 or 10 amino acids, and a C-terminal conserved residue of phenylalanine (F) or leucine (L). Increases were also observed at several other positions: isoleucine (I) and methionine (M) at position 3;

20 aspartic acid (D), glutamic acid (E), glycine (G), lysine (K) and proline (P) at position 4; lysine (K), methionine (M) and asparagine (N) at position 5; valine (V) at position 6; asparagine (N) and valine (V) at position 7; and, alanine (A), glutamic acid (E), lysine (K), glutamine (Q) and serine (S) at

25 position 8.

Table 8.

SummaryHLA-A24.1 Allele-Specific Motif

	Position	Conserved Residues
30	1	-
	2	Y
	3	I, M
35	4	D, E, G, K, P
	5	L, M, N
	6	V
	7	N, V

8	A,E,K,Q,S
9	F,L
10	F,A

5

Example 8Identification of immunogenic peptides

Using the motifs identified above for various MHC class I allele amino acid sequences from various viral and tumor-related proteins were analyzed for the presence of these motifs. Sequences for all of the target antigens were obtained from the GenBank data base (Release No. 71.0; 3/92). The identification of motifs was done using the "FINDPATTERNS" program (Devereux, Haeblerli and Smithes (1984). Nucleic Acids Research 12(1); 387-395).

The amino acid sequence or the nucleotide sequence encoding products was obtained from the GenBank database. In the cases of Human Papilloma Virus (HPV), Prostate Specific antigen (PSA), p53 oncogene, Epstein Barr Nuclear Antigen-1 (EBNA-1), and c-erb2 oncogene (also called HER-2/neu), and Melanoma Antigen-1 (MAGE-1), a single sequence exists.

In the cases of Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), and Human Immunodeficiency Virus (HIV) several strains/isolates exist and many sequences have been placed in GenBank.

For HBV, binding motifs were identified for the adr, adw and ayw types. In order to avoid replication of identical sequences, all of the adr motifs and only those motifs from adw and ayw that are not present in adr were added to the list of peptides.

In the case of HCV, a consensus sequence from residue 1 to residue 782 was derived from 9 viral isolates. Motifs were identified on those regions that had no or very little (one residue) variation between the 9 isolates. The sequences of residues 783 to 3010 from 5 viral isolates were also analyzed. Motifs common to all the isolates were identified and added to the peptide list.

Finally, a consensus sequence for HIV type 1 for North American viral isolates (10-12 viruses) was obtained

from the Los Alamos National Laboratory database (May 1991 release) and analyzed in order to identify motifs that are constant throughout most viral isolates. Motifs that bear a small degree of variation (one residue, in 2 forms) were also added to the peptide list.

Several motifs for each allele shown below were used to screen several antigens. Protein E6 of human papilloma virus (HPV) type 16 using motifs from all of the alleles disclosed above are shown (Table 9). Protein E7 of HPV type 18 was also searched for motifs from all alleles (Table 9) Melanoma antigens MAGE 1, 2 and 3 were searched for motifs from all alleles (Table 10). The antigen PSA was searched for motifs from all alleles (Table 11). Finally, core and envelope proteins from hepatitis C virus were also searched (Table 12). In the tables and the description of the motifs, the conventional symbol letter for each amino acid was used. The letter "X" represents a wild card character (any amino acid).

The following motifs were screened in the present search:

For HLA-A1 (A*0101):

1	XSXXXXXXXXY
2	XSXXXXXXXXXY
25	3 XTXXXXXXXXY
	4 XTXXXXXXXXXY
	5 XXDXXXXXXXXY
	6 XXDXXXXXXXXXY
	7 XXEXXXXXXXXXY
30	8 XXEXXXXXXXXXXY

For HLA-A3.2 (A*0301)

1	XVXXXXXXXXK
2	XVXXXXXXXXK
35	3 XLXXXXXXXXK
	4 XLXXXXXXXXK
	5 XMXXXXXXXXK
	6 XMXXXXXXXXK

50

For HLA-A11 (A*1101):

1 XTXXXXXXXX
2 XTXXXXXXXX
3 XVXXXXXXXX
4 XVXXXXXXXX

5

For HLA-A24.1 (A*2401):

1 XYXXXXXXF
2 XYXXXXXXF
3 XYXXXXXXL
4 XYXXXXXXL

10

Peptides with MHC Class I Binding Motifs Table 9

51

AA Position	Sequence	Antigen	HLA molecule
5	30 IHDIILECVY	HPV16.E6	A1
	69 VCDKCLKFY	HPV16.E6	A1
	77 YSKISEYRHY	HPV16.E6	A1
	80 ISEYRHYCY	HPV16.E6	A1
	92 GTTLEQQYNK	HPV16.E6	A11
	93 TTLEQQYNK	HPV16.E6	A11
	106 LLIRCINCQK	HPV16.E6	A3
10	2 HGDTPTLHEY	HPV16.E7	A1
	16 QPETTDLYCY	HPV16.E7	A1
	44 QAEPDRAHY	HPV16.E7	A1
	89 IVCPICSQK	HPV16.E7	A3, A11
15	3 RFEDPTRRPY	HPV18.E6	A1
	4 FEDPTRRPY	HPV18.E6	A1
	25 LQDIEITCVY	HPV18.E6	A1
	41 LTEVFEFQFK	HPV18.E6	A11
	72 YSRIRELRHY	HPV18.E6	A1
	84 SVYGDITLEK	HPV18.E6	A3, A11
	101 LLIRCLRCQK	HPV18.E6	A3
20	59 HTMLCMCKK	HPV18.E7	A11
25			

Human Papilloma Virus₁₆ and 18 (E6 and E7 Proteins)

52.

Peptides with MHC Class I Binding Motifs Table 10

AA Position	Sequence	Antigen	HLA molecule
5	2 SLEQRS L HCK	MAGE 1	A3
	96 SLFRAVITK	MAGE 1	A3
	96 SLFRAVITKK	MAGE 1	A3
	108 DLVGF L LLK	MAGE 1	A3
	128 MLESVIK N YK	MAGE 1	A3
10	128 MLESVIK N Y	MAGE 1	A1
	152 QLVFGIDVK	MAGE 1	A3
	161 EADPTGHSY	MAGE 1	A1
	182 LLGDNQIMP K	MAGE 1	A3
	215 WEELSVMEVY	MAGE 1	A1
15	223 VYDGREHSAY	MAGE 1	A1
	238 LLTQDLVQEK	MAGE 1	A3
	239 LTQDLVQEK	MAGE 1	A11
	239 LTQDLVQEKY	MAGE 1	A1
	240 TQDLVQEKY	MAGE 1	A1

20

Melanoma Antigen MAGE 1

Peptides with MHC Class I Binding Motifs Table 11

AA Position	Sequence	Antigen	HLA molecule
5	21 IVGGWECEK	PSA	A3, A11
	57 LTAAH CIRNK	PSA	A11
	88 VSHSFPHPLY	PSA	A1
	95 PLYDMSLLK	PSA	A3
	178 DVCAQVHPQK	PSA	A3, A11
10	182 QVHPQKVTK	PSA	A3, A11
	236 PSLYTKVVHY	PSA	A1
	239 YTKVVHYRK	PSA	A11
	241 KVVHYRKWIK	PSA	A3, A11
	242 VVHYRKWIK	PSA	A3, A11

15

Prostate Specific Antigen (PSA)

Peptides with MHC Class I Binding Motifs Table 12

AA Position	Sequence	Antigen	HLA molecule
5	2 STNPKPQRK	HCV	A11
	14 NTNRRPQDVK	HCV	A11
	43 RLGVRATRK	HCV	A3
	302 VQDCNCSIY	HCV	A1
	556 WMNSTGFTK	HCV	A3
10	605 LTPRCMVDY	HCV	A1
	626 FTIFKIRMY	HCV	A1

Hepatitis C Virus (Consensus Sequence)

15

Example 9Quantitative HLA class I binding assay

To verify that motif-containing peptide sequences are indeed capable of binding to the appropriate class I molecules, specific binding assays were established. HLA-A3.2 molecules were purified from GM3107 EBV cells by affinity chromatography using the GAPA3 mAb (anti-A3) to isolate A3.2. Prior to the step, the lysate was depleted of HLA B and C molecules by repeated passages over a B1.23.2 column (this antibody is B,C specific) generally as described in Example 2, above.

As a radiolabeled probe, the peptide 941.12 (KVFPYALINK), containing an A3.2 motif, was used. This peptide contains the anchor residues V₂ and K₁₀, associated with A3.2-specific binders, described above. A Y residue was inserted at position 5 to allow for radiolodination. Peptides were labeled by the use of the Chloramine T method Buus et al., Science 235:1352 (1987), which is incorporated herein by reference.

A dose range of purified A3.2 was incubated with 10 nM of 941.12 at pH 7.0 and 23°C, in presence of a protease inhibitor cocktail (1 mM PMSF, 1.3 mM 1.10 phenanthroline, 73 µM pepstatin A, 8 mM EDTA, and 200 µM N α_p-tosyl-L-lysine chloromethyl ketone (TLCK)), in presence of 1 µM purified human β2 microglobulin. After two days, the % bound radioactivity was measured by gel filtration over TSK 2000 columns as previously described for class II peptide binding assays in Sette et al., in Seminars in Immunology, Vol. 3, Geffer, ed. (W.B. Saunders, Philadelphia, 1991), pp 195-202, which is incorporated herein by reference. (see, Fig. 4). Good binding (in the 60 to 100% range) was observed for A3.2 concentrations ranging between 35 and 300 nM. 30% binding was observed at 15 nM A3.2.

To minimize A3.2 usage and to increase the sensitivity of the assay, a concentration of 5-10 nM A3.2 was selected for further assays. In the experiment shown in Fig. 5, 7nM A3.2 and an equivalent concentration of radiolabeled 941.12 were incubated using the conditions described above and

in the presence of a dose range of three peptides (HBc 18-27 (924.07), a Prostate Specific Antigen peptide (939.01), and HIV nef 73-82 (940.03)). It was found that peptide 940.03 inhibited strongly, with a 50% inhibitory concentration (IC₅₀) of 22 nM, while a weaker inhibition was observed with peptide 939.01 (IC₅₀ 940 nM). Finally, peptide 924.07 did not show any inhibition up to the 30 μ M level. Thus, it is concluded that peptides 940.03 and 939.01 are high and intermediate affinity binders, respectively, while peptide 924.07 is classified as a low affinity or negative binder.

Throughout this disclosure, results have been expressed in terms of IC₅₀'s. Given the conditions in which the assays are run (i.e., limiting MHC and labeled peptide concentrations), these values approximate K_d values. It should be noted that IC₅₀ values can change, often dramatically, if the assay conditions are varied, and depending on the particular reagents used (e.g., Class I preparation, etc.). For example, excessive concentrations of MHC will increase the apparent measured IC₅₀ of a given ligand.

An alternative way of expressing the binding data, to avoid these uncertainties, is as a relative value to a reference peptide. The reference peptide is included in every assay. As a particular assay becomes more, or less, sensitive, the IC₅₀'s of the peptides tested may change somewhat. However, the binding relative to the reference peptide will not change. For example, in an assay run under conditions such that the IC₅₀ of the reference peptide increases 10-fold, all IC₅₀ values will also shift approximately 10-fold. Therefore, to avoid ambiguities, the assessment of whether a peptide is a good, intermediate, weak, or negative binder should be based on its IC₅₀, relative to the IC₅₀ of the standard peptide.

If the IC₅₀ of the standard peptide measured in a particular assay is different from that reported in the table, then it should be understood that the threshold values used to determine good, intermediate, weak, and negative binders should be modified by a corresponding factor. For example, if

in an A2.1 binding assay, the IC₅₀ of the A2.1 standard (941.01) were to be measured as 8 nM instead of 5 nM, then a peptide ligand would be called a good binder only if it had an IC₅₀ of less than 80 nM (i.e., 8nM x 0.1), instead of the usual cut-off value of 50 nM.

The experimental system herein described can be used to test binding of large numbers of synthetic peptides to a variety of different class I specificities. Specific binding assays can be performed as follows.

HLA-A11-specific assay

The cell line BVR was used as a source of HLA. The dependency of the binding on MHC concentration in presence or absence of β_2M are shown in Fig. 6, while Fig. 7 depicts the dose dependency of the inhibition by excess unlabeled ligand. Finally, Fig. 8 shows a Scatchard analysis experiment. Values of apparent K_D of ~6 nM and of 10% active receptor were obtained, and were remarkable for their similarity to the values obtained for A2.1 and A3.2. The sequence of the peptide used as a radiolabeled probe (940-06) is AVDLYHFLK.

HLA-A1-specific assay

In this case, the EBV cell line Steinlin was used as a source of purified HLA. The same protocol previously applied to purification of other HLA alleles (i.e., depletion of B, C molecules by a B1.23.2 mAb column, followed by purification of A molecules by means of a W632 mAb column) was utilized. On the basis of the pool sequencing data, consensus peptides were synthesized, directly radiolabeled, and tested for HLA binding using the standard protocol (1 mM β_2M , 2 days RT incubation in presence of protease inhibitors). A graph illustrating the relationship between % binding and μM input HLA A1 is shown in Fig. 9. From the data, it was concluded that in analogy with what was observed for HLA A2, 3, and 11, as little as 30 nM are sufficient to obtain ~10% binding. The sequence of the peptide used as a radiolabeled probe (944.02) is YLEPAIAKY. In the next set of experiments, the specificity of the assay established was verified by its inhabitability by excess unlabeled peptide. The IC₅₀ was measured (Fig. 10) as

20 nM. Further Scatchard analysis (Fig. 11) verified that the apparent K_D of the interaction corresponded to 21 nM, with a % of active receptor corresponding to 5.1%.

5

HLA-A24 specific assay

HLA A24 molecules were purified from the KT3 EBV cell line. In this case, two consensus peptides whose sequences were based on the pool sequencing data have been synthesized. Their sequences are: 979-01, AYIDNVYKF and 979.02, AYIDNYNKF. The results of experiments in which the % bound of these two peptides as a function of input MHC was measured are shown in Fig. 12. In both cases, 10-15% binding was obtained with as little as 20-50 nM MHC. Cold inhibition experiments (Fig. 13), limiting MHC concentrations, revealed that the binding was readily inhibitable by excess unlabeled peptide, with an apparent K_D of 30 and 60 nM, respectively. Further Scatchard experiments verified values of 136 nM and 28 nM, respectively. The apparent % of available receptor (active MHC) were 8.3% and 7.4%, respectively (Fig. 9a and b). On the basis of these data, peptide 979.02 was arbitrarily selected as standard label indicator for A24 assays. Furthermore, on the basis of the data herein described, we also conclude that the goal of establishing an A24-specific binding assay has been accomplished. In conclusion, specific assays for the five major HLA alleles have been described.

Example 10

Expansion of HLA A Motifs

Establishing *in vitro* binding assays allows one to readily quantitate *in vitro* the binding capacity of various synthetic peptides to the various alleles of interest (HLA A1, A2, A3, A11, and A24). This allows verification of the correctness of the motifs by means of peptides carrying the various HLA A motifs for their capacity to bind purified HLA molecules. Typically, peptides were synthesized with specific HLA motifs embedded in a neutral backbone composed of only alanine residues. In some cases, a K residue was also introduced within the sequence, with the purpose of increasing

solubility. The use of such "neutral" poly A backbones, as applied to the case of class II molecules, has been described in detail, for example, by Jardetzky et al. (Jardetzky et al., EMBO J. 9(6):1797,1990).

5 For example, in the case of A3.2, a motif has been defined with a hydrophobic residue in position 2 and a positive charge (K) in position 9. Thus, to verify that the presence of these two anchor residues would allow, in the context of a poly A backbone, for A3.2 binding, the poly A
10 analog with the sequence AMAAAAAAK was synthesized (Table 13).

Similarly, other peptides carrying other HLA motifs were also synthesized and tested for HLA binding. It was found that in all cases, the presence of the specific HLA motifs was conducive to binding to the relevant HLA allele,
15 with estimated K_D comprised of between 125 and 2.8 nM. In most cases, the binding was also absolutely specific, in that no binding was detected to irrelevant alleles. Only two exceptions to this general rule were observed. Firstly, A3 and A11 peptides crossreacted extensively with each other,
20 perhaps as could have been expected by the fact that the motifs for these two alleles are remarkably similar. Second, some A1 peptides crossreacted, albeit with much lower affinities, on A11 and A3.2.

To further define the structural requirements for the
25 interaction between peptide epitopes and various class I alleles of interest, analogs of 10 residues in length of some of the 9 residue peptides shown in Table 13 were synthesized (Table 14). These analogs were generated by inserting an additional Ala residue within the poly A backbone, so that the
30 anchor residues are not located in positions 2 and 10 (as opposed to 2 and 9 in the previous Table). The results obtained illustrate that motifs of 10 residues are also capable of specifically binding to the relevant class I alleles, albeit with a slightly lower efficiency.

35 In summary, these data confirm that both 9-mer and 10-mer peptides which contain the appropriate motifs can bind HLA. On the basis of these data, 8-mer or 11-mer peptides

should also be capable of binding, even if perhaps with lower affinities.

The data described above show that the presence of certain residues in the anchor positions does allow (at least in a "neutral" poly A backbone) for HLA binding. To investigate to what degree other amino acids (for example, chemically related amino acids) might be tolerated in these crucial anchor positions, analogs of some of the poly A peptides from Table 13 were synthesized, in which the residue present in position 2 (or 3) or 9 was varied. The results of this analysis are shown in Tables 15-19.

In the case of A3.2 (Table 15), in position 2, L, M, I, V, S, A, T, and F were found to be preferred (binding ≥ 0.1 relative to previously defined anchor residues), while C, G, and D were permitted (binding ≥ 0.01 to 0.1 relative to previously defined anchor residues). The substitution of E, because of its similarity to D, in this position should also be tolerated. In position 9, K, R, and Y were preferred. Because of a similarity in nature, that H and F should also be preferred. No other residue was tolerated in position 9 for A3 binding.

In the case of A11 (Table 16), the preferred residues in position 2 were L, M, I, V, A, S, T, G, N (L and Q by similarity). Tolerated were C, F, D (and E by similarity). In position 9, K was preferred and R was tolerated. H should also be tolerated by similarity.

In the case of A24 (Table 17), Y and F were preferred in position 2 (and W by similarity); no other residue was tolerated. In position 9, F, I, and L were preferred (and W and M by extension). No other residue was tolerated.

In the case of A1, three different anchor residues had previously been defined. The results shown in the preceding section show that they act independently of each other (i.e., that two out of three anchors would be sufficient for binding). This is indeed the case. For this reason, analogs containing two anchors were synthesized to define what residues might be preferred or tolerated in each position. The data shown in Table 18 show that in position 2, T, S, and

M are preferred, and no other residue is tolerated. In position 3 (Table 19), D and E are preferred, and A, S (and T by similarity) are tolerated. Finally, in position 9, only Y is preferred, and no other residue appears to be tolerated (Table 19).

Thus, on the basis of the data, it is concluded that peptides carrying any combination of two preferred residues can bind. Peptides containing "imperfect" motifs, i.e., carrying a preferred residue at one position and a tolerated one at the other anchor position, should also be capable of binding, even if with somewhat lower affinity. Using the motifs of this invention for various MHC class I alleles amino acid sequences from various viral and tumor-related proteins were analyzed for the presence of motifs. The results of this motif analysis is shown in Table 23 a - k.

Example 11

Validation of HLA Peptide Binding Motifs with an Unbiased Set of HPV 16 Peptides.

Human Papillomaviruses (HPVs) are implicated in the etiology of cervical cancer (Pfister, H. (1974) *Biology and biochemistry of papillomaviruses*, Rev. *Physiol. Biochem.* 99:111; zur Hausen, H. (1991). Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology*. 184:9) and in up to 10% of total mortality due to cancer worldwide (zur Hausen, H. (1991). *Viruses in Human Cancers*. *Science*, 254:1167). Cervical cancer is the second most common cause of cancer-related death in females worldwide (Parkin, D. M., Laara, E., and Muir, C. S. (1988), Estimates of the worldwide frequency of sixteen major cancers in (1980). *Int. J. Cancer*. 41:184). HPV DNA is present in more than 90% of the cervical carcinomas and predominantly of the HPV 16 genotype (Resnick, R. M., Cornelissen, M. T., Wright, D. K., Eichinger, G. H., Fox, H. S., ter Schegget, J., and Manos, M. M. (1990). Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. *J. Natl. Cancer Inst*; Van den Brule, A. J. C., Walboomers, J. M. M., du Maine, M., Kenemans, P., and Meijer,

- C. J. L. M. (1991). Difference in prevalence of human papillomavirus genotypes in cytomorphologically normal smears is associated with a history of cervical intraepithelial neoplasia. *Int. J. Cancer*. 48:404). The ability of HPV 16 early region 6 and 7 (E6, E7) open reading frames to in vitro immortalize rodent cells (Yasumoto, S., Burkhardt, A.L., Doniger, J., and DiPaolo, J.A. (1986). Human Papillomaviruses type 16 DNA induced malignant transformation of NIH3T3 cells. *J. Virol.* 57:572) and human keratinocytes (Pirisi, L., Yasumoto, S., Feller, M., Doniger, J., and DiPaolo, J.A. (1987). Trans-formation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *J. Virol.* 61:1061) and to transform human fibroblasts (Smits, H. L., Raadsheer, E., Rood, I., Mehendale, S., Slater, R. M., van der Noordaa, J., and ter Schegget, J. (1988). Induction of anchorage-independent growth of human embryonic fibroblasts with a deletion in the short arm of chromosome 11. *J. Virol.* 62:4538) suggests direct involvement of HPV 16 in the multi-step process of cervical carcinogenesis,
- 20 In general T cell immunity, in particular mediated by cytotoxic T lymphocytes (CTL) is important in the defense against virus-induced tumors (Melief, C. J. (1992). Tumor eradication by adoptive transfer of cytotoxic T lymphocytes. *Adv. Cancer Res.* 58:143; Melief, C. J., and Kast, W. M. (1992). Lessons from T cell responses to virus induced tumors for cancer eradication in general. *Cancer Surv.* 13:81). Recently in a mouse model, it was reported that some degree of protection against HPV 16 E7 expressing tumors can be obtained with CTL after immunization with HPV 16 E7 expressing cells
- 30 (Chen, L., Thomas, E, K., Hu, S. L., Hellström, I., and Hellstrom, K. E. (1991). Human papillomavirus type 16 nucleoprotein E7 is a tumor rejection antigen. *Proc. Natl. Acad. Sci.* 88:110; Chen, L., Ashe, S., Brady, W. A., Hellstrom, I., Hellström, K. E., Ledbetter, J. A., McGowan, P., and Linsley, P. S. (1992). Costimulation of Antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell.* 71:1093). In vivo protection by CTL was recently shown in mouse models in which

synthetic peptides containing CTL epitopes were used for efficient priming of mice against virus infections (Schulz, M., Zinkernagel, R. M., and Hengartner, H. (1991).

Peptide-induced antiviral protection by cytotoxic T cells.

- 5 Proc. Natl. Acad. Sci. USA 88:991; Kast, W. M., Roux, L., Curren, J., Blom, H. J. J., Voordouw, A. C., Meleon, R. H., Kolakofski, D., and Melief, C. J. M. (1991). Protection against lethal Sendai virus infection by in vivo priming of virus-specific cytotoxic T lymphocytes with an unbound
10 peptide. Proc. Natl. Acad. Sci. USA. 88:2283). Moreover in a mouse model it has now been shown that complete protection against HPV 16 induced tumors can be achieved by peptide vaccination with a CTL epitope derived from the viral oncogene E7().

- 15 The HPV 16 E6 and E7 gene products are the most desirable target antigens for vaccination against HPV 16 induced tumors. Both are retained and highly expressed in HPV 16-transformed cancer cells in vivo (Baker, C. J., Phelps, W. C., Lindgren, V., Braun, M. J., Gonda, M. A., and Howley, P. M. [1987]. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell
20 lines. J. Virol. 61:962; Smotkin, D., and Wettstein, F. O. [1986]. Transcription of human papillomavirus type 16 early genes in a cervical cancer and cancer-derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA.
25 83:4680) and involved in the induction and maintenance of cellular transformation in vitro (Crook, T., Morgenstern, J. P., Crawford, L., and Banks, L. [1989]. Continued expression of HPV-16 E7 protein is required for maintenance of the transformed phenotype of cells co-transformed by HPV-16 plus
30 EJ ras. EMBO J. 8:513; Hawley-Nelson, P., Vousden, K. H., Hubbert, N. L., Lowy, D. R., and Schiller, J. T. [1989]. HPV 16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. EMBO J. 8:3905). Dependence of in vitro growth
35 of cell lines derived from cervical cancers on the expression of E6 and E7 emphasizes involvement of these oncogenes in maintenance of the phenotype of cervical carcinoma cell lines (Von Knebel Doeberitz, M., Bauknect, T., Bartch, D., and zur

Hausen, H. [1991]. Influence of chromosomal integration on glucocorticoid-regulated transcription of growth-stimulation papillomavirus genes E6 and E7 in cervical carcinoma cells. *Proc. Natl. Acad. Sci. USA.* 88:1411). To determine the CTL epitopes and potential vaccine candidates of HPV 16 for humans, we screened peptides spanning the HPV 16 E6 and E7 protein sequences for their ability to bind to the most frequent human MHC molecules, namely HLA-A1, A3.2, A11.2 and A24. Combined these five alleles will cover about 90% of the world population (Dupont, B., ed. [1987]. *Immunology of HLA Vol. I -- Histocompatibility Testing.* Springer-Verlag, New York).

A complete set of 240 overlapping synthetic peptides of 9 aa length and 8 aa overlap covering the entire HPV 16 E6 and E7 oncogene sequences were synthesized. The peptides were tested for their ability to bind the aforementioned HLA molecules in the binding assay described above. The results of this analysis show the relative affinity of all peptides for the respective HLA alleles and reveal the possible candidate CTL epitopes for use in peptide based vaccines for humans in Tables 20(a)-(d).

The results confirm that peptide binding motif described in this invention for the aforementioned HLA alleles predict which peptide of a protein is likely to bind into the groove of a specified HLA molecule. Since we used a large and unbiased set of peptides, the results of the peptide binding analyses were used to evaluate the value of these motifs both for their predictive capacities and the necessity to have particular anchor aa residues on positions 2, (3) and 9 in a peptide.

Peptides. Peptides were generated by solid phase strategies on a multiple peptide synthesizer (Abimed AMS 422) by repeated cycles in which addition of Fmoc protected amino acids to a resin of polystyrene was alternated with a Fmoc-deprotection procedure (Gausepohl, H., Kraft, M., Boulin, Ch., and Frank, R. W. [1990]. Automated multiple peptide synthesis with BOP activation. in *Proc. of the 11th American peptide symposium.* J. E. Rivier and G. R. Marshall, Ed.

ESCOM, Leiden. 1003-1004). The peptides all carrying a COOH group at the C-terminal end, were cleaved from the resin and side chain protective groups were removed by treatment with aqueous TFA. Peptides were analyzed by reversed phase HPLC lyophilized and dissolved at a concentration of 1 mg/ml in phosphate-buffered saline with 3% DMSO (Sigma, St. Louis, MO 63175) before use. Once dissolved, the peptides were stored at -70° C. Since cysteine containing peptides are susceptible to (air) oxidation during synthesis and handling, these peptides were synthesized with an alanine instead of a cysteine.

Identification of peptides from HPV 16 E6 and E7 proteins that bind to different HLA-A alleles. A complete set of 240 peptides of 9 aa in length and overlapping by 8 aa, covering the sequences of the entire HPV 16 E6 and E7 proteins, was tested for binding to 5 different HLA-A molecules.

The results of this analysis are depicted in Tables 20(a)-(d). Table 20(a) describes the peptides of HPV 16 that bound to HLA-A1 molecules. All peptides were tested. Listed are only peptides yielding ratio values of ≥ 0.001 . It can be seen that 2 peptides bound with high affinity to this molecule (>0.1), 6 with intermediate affinity (0.1-0.01) and 1 with low affinity (0.01-0.001). Peptides were ranked by ratio value to allow comparison of data obtained in different experiments. To calculate the concentration of a peptide necessary to yield a 50% inhibition dose (IC_{50}) one has to divide the value of the standard IC_{50} by the ratio. For example, peptide E6-80 has an IC_{50} of 23 nM (81/3.5).

Table 20(b) describes the peptides that bound to HLA-A3.2 molecules. Seven peptides were identified as high affinity binders, 6 as intermediate affinity binders and 13 as low affinity binders. Table 20(c) describes the peptides that bound to HLA-A11.2 molecules. Six high affinity peptides were identified, 4 intermediate affinity binders and 10 low affinity binders. Two high affinity binding peptides (E6-59 IVYRDGNPY and E6-80 ISEYRHYAY) and two weak affinity binding peptides with a Y at the 9th position (E6-42 QQLLRREYV, E6-69

VADKALKFY) were identified for HLA-A11.2. Considering the high binding strength of the first two peptides and the similarity between the HLA-A11.2 motif and the HLA-A3.2 motif in which Y's are preferred at the 9th aa position, tyrosines should be included at the 9th position in the HLA-A11.2 motif. Comparing Tables 21(b) and (c) it is clear that there is a large overlap of peptides that bound to both A3.2 and A11.2 molecules. Eighteen out of 28 E6 and E7 peptides binding to these two HLA molecules overlapped and only 8 peptides were unique for HLA-A3.2 and 2 peptides unique for HLA-A11.2.

Finally, Table 20(d) describes the peptides that bound to HLA-A24 molecules. Here 2 peptides were identified as high affinity binding peptides, 5 as intermediate affinity binding peptides and 5 as low binding peptides. One high affinity peptide (E6-72 KALKFYSKI) and one intermediate affinity peptide (E7-49 RAHYNIVTF) were identified, indicating that an A at the second position should be allowed in the HLA-A24 motif. All these inclusions are indicated in Table 20-e. In analyzing these tables it can be concluded that between 2 and 7 high affinity binding peptides were identified for all of the tested HLA-A molecules. Occasionally some peptides were binding to more alleles. Three peptides (E6-7, E6-37 and E6-79), bound to HLA-A2.1, A3.2 and A11.2. One peptide (E6-38) bound to HLA-A3.2, A11.2 and A24 and two peptides (E6-69 and E6-80) bound to HLA-A1, A3.2 and A11.2. But these crossreactive peptides bound only weakly to one or more of the different HLA molecules. In general, however, it can be concluded that, except for HLA-A3.2 and HLA-A11.2 molecules, almost all HLA molecules bind unique peptides.

Validation of HLA-A peptide binding motifs with an unbiased set of HPV 16 E6 and E7 peptides.

We analyzed how well the motifs for anchor positions described in this invention predicted the binding of a peptide, and also the reverse: how well binding peptides followed the identified motifs. For this, peptides were ranked as high binders, intermediate binders, weak binders, and negative binders and for each peptide the motif prediction based on the anchor motif rules of Table 6 were analyzed. The

overall efficiency of the 2, (3), and 9 anchor motifs was then calculated and this is summarized in Table 20(e). It can be concluded that the motif described above for the different HLA-A molecules are quite accurate. One hundred percent of the HLA-A1, A3.2, and A24 high binders would be predicted as well as 67% of the HLA-11.2. Even for the intermediate binders between 40 and 100% would be predicted depending on the HLA-A molecule analyzed. Furthermore, the percent of weak binding peptides that would be predicted is low and the percent of those peptides that were predicted to bind but actually did not bind is very low for all these alleles.

Analyzed differently, of the 12 peptides predicted to bind to HLA-A1 actually 5 bound with high or intermediate affinity. This indicates that only a few peptides would have to be made to find these potential CTL epitopes. The figures for HLA-A3.2, A11.2, and A24 were 10/32, 7/26, and 4/7, respectively. This implies that the predictive value for all of these alleles is good. Besides a small number of peptides that had not been predicted by the recently described motifs, the (-) in Tables 21(a) - (d), a number of peptides that were predicted by the 2, (3) and 9 anchor motifs did not bind, indicating that having the right anchor residues is not always sufficient for binding and implicating that non-anchor residues can make negative contributions to the binding of a peptide.

Example 12

Presence of a Motif is Necessary But Not Sufficient for High Affinity Class I Binding

To investigate further how the presence of different motifs might influence the capacity of different peptides to bind to the relevant HLA alleles, the sequences of various potential target molecules were scanned for the presence of motif-containing peptides. The peptides thus identified were synthesized and tested for binding. It was found (Table 20) that in the case of A3.2, only 39 (19%) of the 205 peptides bound with high affinity in the 1 to 50 nM range. 22.4% of them bound with intermediate affinities (in the 50 to 500 nM

range), while 34.6% bound weakly (in the 500 nM to 50 μ M range). Finally, 23.9% of them did not bind at all, at least up to the 50 μ M level. In the case of A11, 33 (33%) of the 100 peptides bound with high affinity in the 1 to 50 nM range. 5 35% of them bound with intermediate affinities (in the 50 nM range.), while 24% bound weakly (in the 500 nM to 50 μ M range). Finally, 8% of them did not bind at all, at least up to the 50 μ M level.

Similar results were also obtained (data not shown) 10 in the case of A1 and A24.

The same type of analysis were also performed in the case of 10-mer peptides carrying either the A3.2, and A11 motifs (Tables 22(a) and (b)). It was found that in these cases, the frequency of good binders was even lower (17.5%, 15 and 29.8%, respectively). These data confirm the fact that motif-containing 10-mer peptides can indeed bind, albeit with, in general, reduced affinity.

In summary, the data shown in this section clearly show that the presence of the correct anchor residues is not 20 sufficient per se to allow for good HLA binding. It is thus apparent that the nature of the residues contained in positions other than 2(3) and 9 (or 10) can influence binding. The most likely explanation of this observation is that the presence of certain residues (in positions other than 2 and 9) 25 can negate or increase the binding potential of a peptide determinant.

The data shown in the preceding sections describe how specific binding assays can be used to identify, within motif-containing peptides, peptides that are immunogenic. We also 30 wanted to devise an alternative strategy, namely to derive procedures that would be able to predict, within motif-containing peptides, which peptides might be good or intermediate binders and thereby might be immunogenic. In other experiments not shown intermediate or good binders have 35 been shown to be immunogenic. In particular, to identify residues that have a negative impact on binding an analysis of all positions for A3.2, A11, and all motif-containing peptides, both 9-mers and 10-mers is carried out. In the case

of A11, because of the small occurrence of nonbinding peptides, a different cutoff was used such that the analysis compares good and intermediate binders on the one hand to weak and nonbinders on the other.

5

Example 13

Algorithms to Identify Immunogenic Peptides

In light of results presented in Example 13 above, algorithms are developed to provide a more exact predictor of binding based upon the effects of different residues at each position of a peptide sequence, in addition to the anchor or conserved residues. More specifically, we utilize the data bank obtained during the screening of our collection of A1, 3,11 or 24 motif containing peptides to develop an algorithm for each particular allele which assigns a score for each amino acid at each position along a peptide. The score for each residue is taken as the ratio of the frequency of that residue in good and intermediate binders to the frequency of occurrence of that residue in non-binders.

In the present algorithm residues have been grouped by similarity. This avoids the problem encountered with some rare residues, such as tryptophan, where there are too few occurrences to obtain a statistically significant ratio. A listing is made of scores obtained by grouping for each of the twenty amino acids by position for 9-mer peptides containing conserved residues that define their motif (2/9 motifs). A peptide is scored in the algorithm as a product of the scores of each of its residues.

The power of an algorithm to correlate with binding is further underlined by its ability to predict a population of peptides with the highest occurrence of good binders. If one were to rely, for example, solely on the 2/9 motif for predicting 9-mer peptides which bind to a specific MHC allele the large number of peptides containing the motif would be predicted to be good binders. In fact only a relatively small percentage of these peptides are good binders and a somewhat larger percentage are intermediate binders, while a still larger percentage of the peptides predicted by the motif are

either weak or non-binding peptides. In contrast, using the grouped algorithm of this invention a population of peptides are created with a greater percentage of good binders, a still greater percentage of intermediate binders, and a smaller percentage, relative to that predicted by motif-containing peptides, are weak and non-binders.

The present example of an algorithm uses the ratio of the frequency of occurrence of an amino acid in binders and non-binders to measure the impact of a particular residue at each position of a peptide. It is immediately apparent to one of ordinary skill in the art that there are alternative ways of creating a similar algorithm. For example, one could use average binding affinity values, or relative binding of single amino acid substitutions in a motif containing peptide with a poly-alanine backbone to generate an algorithm table.

An algorithm using average binding affinity has the advantage of including all of the peptides in the analysis, and not just good/intermediate binders and non-binders. Moreover, it gives a more quantitative measure of affinity than the simpler group ratio algorithm. We create such an algorithm by calculating for each amino acid, by position, the average log of binding when that particular residue occurs in our set of motif containing peptides. The algorithm score for a peptide is then taken as the sum of the scores by position for each of its residues.

Example 14

Preparation of effective HLA allele-specific antigen presenting cells.

This example demonstrates the use of cold temperature incubation or acid stripping/peptide loading method to prepare effective HLA-allele-specific antigen presenting cells (APC). The APC were used to sensitize precursor cytotoxic T lymphocytes which led to the development of antigen-specific cytotoxic cells. This was accomplished using either phytohemagglutinin (PHA) T-cell blasts or peripheral blood mononuclear cells (PBMC) or staphylococcus aureus Cowan I (SAC-I) activated PBMC as APC. The results are applicable to other APC and to the other MHC alleles.

The following describes sources for materials used in the following examples:

- L-Ascorbic acid, Cat #B582, J.T. Baker, Phillipsburg, NJ.
- 5 Anti-HLA A2 (BB7.2), Cat #HB82, ATCC, Rockville, MD.
Anti-HLA DR (LB3.1), from J. Gorga, Children's Hospital, Pittsburgh, PA.
Anti-HLA Alpha chain pan ABC (9.12.1), from R. DeMars, University of Wisconsin, Madison, WI.
- 10 Anti-mouse IgG FITC conjugate, Cat #F2883, Sigma, St. Louis, MO.
 β_2 microglobulin, Cat #MO114, Scripps Labs, San Diego, CA.
BSA Fraction V, Cat #A9418, Sigma, St. Louis, MO.
- 15 50cc conical centrifuge tubes, Cat #2070, Falcon, Lincoln, Park, NJ.
Cryo 1°C freezing container, Cat #5100-0001, Nalge, Rochester, NY.
Cryovial, Cat #5000-0012, Nalge, Rochester, NY.
- 20 Dimethyl sulfoxide (DMSO), Cat #D2650, Sigma, St. Louis, MO.
DNase, Cat #260912, Calbiochem, San Diego, CA.
Dynabeads M-450 goat anti-mouse IgG, Cat #110.06, Dynal, Great Neck, NY.
- 25 EDTA tetrasodium salt, Cat #ED4SS, Sigma, St. Louis, MO.
FACScan, Becton Dickinson, San Jose, CA.
Fetal calf serum (FCS), Cat #3000, Irvine Scientific, Irvine, CA.
- 30 Ficoll-Paque, Cat #17-0840-03, Pharmacia, Piscataway, NJ.
Gentamicin, Cat #600-5750AD, Gibco, Grand Island, NY.
L-Glutamine, Cat #9317, Irvine Scientific, Irvine, CA.
- 35 GS-6KR centrifuge, Beckman Instruments, Palo Alto, CA.
Human AB serum (HS), Cat #100-112, Gemini Bioproducts, Calabasas, CA.

- Human rIL-2, Sandoz, Basel, Switzerland.
Human rIL-7, Cat #F1-1587-1, Genzyme, Cambridge, MA.
Isopropanol, Cat #A464-4, Fisher Scientific,
Pittsburgh, PA.
- 5 MicroCELLector T-150 culture flask for selection of
CD4+ cells, Cat #8030, Applied Immune Sciences, Menlo
Park, CA.
Micromedic automatic gamma counter, ICN Micromedics
Systems, Huntsville, AL.
- 10 OKT4 hybridoma supernatant, Cat #CRL 8002, ATCC,
Rockville, MD.
Paraformaldehyde, Cat #T-353, Fisher, Pittsburgh, PA.
PBS calcium and magnesium free (CMF), Cat #17-516B,
BioWhittaker, Walkersville, MD.
- 15 Peptides used in this study were synthesized at Cytel
and described in Table 24 a.
Phytohemagglutinin (PHA), Cat #HA-16, Wellcome,
Dartford, England.
RPMI 1640 + Hepes + glutamine, Cat #12-115B,
BioWhittaker, Walkersville, MD.
- 20 RPMI 1640 + Hepes + glutamine, Cat #380-2400AJ,
Gibco, Grand Island, NY.
Sodium chloride (NaCl), Cat #3624-05, J.T. Baker,
Phillipsburg, NJ.
- 25 Sodium (⁵¹Cr) chromate, Cat #NEZ 030, NEN,
Wilmington, DE.
Sodium phosphate monobasic, Cat #S9638, Sigma, St.
Louis, MO.
Triton X-100, Cat #X-100, Sigma, St. Louis, MO.
- 30 24 well tissue culture plate, Cat #3047, Falcon,
Becton Dickinson, San Jose, CA.
96 well U-bottomed cluster plate, Cat #3799, Costar,
Cambridge, MA.
- 35 *Culture Medium.* PHA blasts and CTL inductions were done in
RPMI 1640 + Hepes + glutamine (Gibco) supplemented with 2 mM
L-glutamine (Irvine Scientific), 50 µg/ml gentamicin (Gibco),
and 5% heat inactivated pooled human Type AB serum (Gemini

Bioproducts) [RPMI/5% HS]. EBV transformed lymphoblastoid cell lines (LCL) were maintained in RPMI 1640 + Hepes + glutamine (BioWhittaker) supplemented with L-glutamine and gentamicin as above and 10% heat inactivated fetal calf serum (Irvine Scientific) [RPMI/10% FCS]. Chromium release assays were performed in RPMI/10% FCS.

Cytokines. Recombinant human interleukin-2 (rIL-2) (Sandoz) was used at a final concentration of 10 U/ml. Recombinant human interleukin-7 (rIL-7) (Genzyme) was used at a final concentration of 10 ng/ml.

Isolation of Peripheral Blood Mononuclear Cells (PBMC). Whole blood was collected in heparin (10 U/ml) containing syringes and spun in 50cc conical centrifuge tubes (Falcon) at 1600 rpm (Beckman GS-6KR) 15 min. The plasma layer was then removed and 10 ml of the buffy coat collected with a 10 ml pipette using a circular motion. The buffy coat was mixed thoroughly and diluted with an equal volume of serum free RPMI 1640. The diluted buffy coat was then layered over 20 ml Ficoll-Paque (Pharmacia) in a 50cc conical tube and centrifuged 400 x g for 20 min at room temperature with the brake off. The Ficoll-plasma interface containing the PBMCs was collected using a transfer pipet (two interfaces per 50cc tube) and washed three times with 50 ml RPMI (1700, 1500, and 1300 rpm for 10 min).

Freezing and Thawing PBMC. PBMC were frozen at 30×10^6 cells/ml of 90% FCS + 10% DMSO (Sigma), in 1 ml aliquots using cryovials (Nalge). Cryovials were placed in Cryo 1°C freezing containers (Nalge) containing isopropanol (Fisher) and placed at -70°C from 4 hr (minimum) to overnight (maximum). Isopropanol was changed after every 5 uses. Cryovials were transferred to liquid nitrogen for long term storage. PBMC were thawed by continuous shaking in a 37°C water bath until the last crystal was nearly thawed. Cells were immediately diluted into serum free RPMI medium containing DNase 30 µg/ml (to avoid clumping) (Calbiochem), and washed twice.

Depletion of Lymphocyte Subpopulations. CD4 lymphocyte depletion was performed using antibody-coated flasks: MicroCELLector T-150 flasks for the selection of CD4+ cells (Applied Immune Sciences) were washed according to the manufacturer's instructions with 25 ml PBS CMF + 1 mM EDTA (Sigma) by swirling flasks for 30 sec followed by incubation for 1 hr at room temperature on a flat surface. Buffer was aspirated and flasks were washed 2 additional times by shaking the flasks for 30 sec and maintaining coverage of the binding surface. To each washed flask, 25 ml culture medium + 5% HS were added and incubated for 20 min at room temperature on a flat surface. Media was left in the flask until it was ready to receive the cells. PBMC were thawed in RPMI/5% HS containing 30 µg/ml DNase, and washed twice. HS in the wash blocks Fc receptors on PBMCs. For one flask a maximum of 12×10^7 cells were resuspended in 25 ml culture medium. Culture medium was aspirated from the flask and then the cell suspension was gently added to the MicroCELLector. Flasks containing the cells were incubated for 1 hr at room temperature on a flat surface. At the end of the incubation, the flask was gently rocked from side to side for 10 sec to resuspend the nonadherent cells. Nonadherent CD4 depleted cells were harvested, and then flasks were washed twice with PBS CMF to collect the nonadherent cells. Harvested CD4-depleted cells were pelleted by centrifugation and resuspended in complete culture medium (RPMI/5% HS).

Generation of PHA Blasts. PBMC were isolated using the standard Ficoll-Paque protocol. Frozen cells were washed twice before use. Cells were cultured at 2×10^6 /ml in RPMI/5% HS containing 1 µg/ml PHA (Wellcome) and 10 U/ml rIL-2. PHA blasts were maintained in culture medium containing 10 U/ml r IL-2 with feeding and splitting as needed. PHA blasts were used as APC on day 6 of culture.

Generation of empty class I molecules and peptide loading were only performed by the acid strip method when using these APC.

Acid Stripping/Peptide Loading of PBMC and PHA Blasts. PBMC were isolated using the Ficoll-Paque protocol. When using

frozen cells, PBMC were washed twice before using. PHA blasts were prepared as previously described and washed twice before using. Once cells were prepared, they were washed once in cold sterile 0.9% NaCl (J.T. Baker) + 1% BSA. In a 50cc
5 conical centrifuge tube, the cells were resuspended at 10^7 /ml in cold sterile citrate-phosphate buffer [0-13 M L-ascorbic acid (J.T. Baker), 0.06 M sodium phosphate monobasic (Sigma) pH 3, 1% BSA, 3 μ g/ml β_2 microglobulin (Scripps Labs)] and incubated for 2 min on ice. Immediately, 5 volumes of cold
10 sterile neutralizing buffer #1 [0.15 M sodium phosphate monobasic pH 7.5, 1% BSA, 3 μ g/ml β_2 microglobulin, 10 μ g/ml peptide] were added, and the cells were pelleted at 1500 rpm, 5 min at 4°C. Cells were resuspended in 1 volume cold sterile neutralizing buffer #2 [PBS CMF, 1% BSA, 30 lig/ml DNase, 3
15 μ g/ml β_2 microglobulin, 40 μ g/ml peptide] and incubated for 4 hrs at 20°C. Cells were diluted with culture medium to approximately 5×10^6 /ml and irradiated with 6000 rads. Cells were then centrifuged at 1500 rpm for 5 min at room temperature and resuspended in culture medium. The acid
20 stripped/peptide loaded cells were used immediately in the CTL induction cultures (below).

Induction of Primary CTL using Acid Stripped/Peptide Loaded Autologous PBMCs or PHA Blasts as Stimulators. Acid
25 stripping/peptide loading of PBMC and PHA blasts are described above. During the last 4 hr incubation of stimulator cells with peptide, the responder cell population was prepared: Responders were PBMC that were depleted of CD4+ cells (described above). Responder cells were resuspended in
30 culture medium at 3×10^6 /ml. 1 ml of the responder cell suspension was dispensed into each well of a 24-well tissue culture plate (Falcon, Becton Dickinson). The plates were placed in the incubator at 37°C, 5% CO₂ until the stimulator population was ready. Once irradiated, stimulator APC were
35 resuspended in culture medium containing 20 ng/ml rIL-7 at 10^6 /ml for the PBMC, or at 3×10^5 /ml for the PHA blasts. 1 ml of stimulator cell suspension was added per well to the plates containing the responders. On day 7 after induction, a

100 μ l culture medium containing 200 ng/ml rIL-7 was added to each well (20 ng/well rIL-7 final). On day 10 after induction, 100 μ l of culture medium containing 200 U/ml rIL-2 was added to each well (20 U/well rIL-2 final).

5

Antigen Restimulation of CTL. On day 12-14 after the induction, the primary CTL were restimulated with peptide using adherent APC. Autologous PBMC were thawed and washed as described above. Cells were irradiated at 6000 rads. Cells were pelleted and resuspended in culture medium at 4×10^6 /ml. 1 ml of cell suspension was added to each well of a 24-well tissue culture plate, and incubated for 2 hrs at 37°C, 5% CO₂. Non-adherent cells were removed by washing each well three times with serum free RPMI. After this step, a 0.5 ml culture medium containing 3 μ g/ml β_2 microglobulin and 20 μ g/ml total peptide was added to each well. APC were incubated for 2 hrs at 37°C, under 5% CO₂ with the peptide and β_2 microglobulin. Wells were aspirated and 1 ml of responder cells at 1.5×10^6 /ml in culture medium was added to each well. After 2 days, 1 ml of culture medium containing 20 U/ml rIL-2 was added to each well.

25

Cytotoxicity Chromium Release Assay. Seven days following restimulation of primary induction, the cytotoxic activity of the cultures was assessed.

30

a. Effector Cell Preparation: the responders, which at this stage are renamed "effectors", were centrifuged and resuspended at 10^7 /ml in RPMI/10% FCS. Three-fold serial dilutions of effectors were performed to yield effector to target ratios of 100:1, 33:1, 11:1, and 3:1. Effector cells were aliquoted at 100 μ l/well on 96 well U-bottomed cluster plates (Costar), in duplicate.

35

b. Target Cell Preparation: Approximately 16-20 hrs prior to the assay, target cells were resuspended at 3×10^5 /ml in RPMI/10% FCS in the presence or absence of 3 μ g/ml β_2 microglobulin and 10 μ g/ml total peptide. After preincubation, target cells were centrifuged and pellets were resuspended in 200 μ l (300 μ Ci) sodium (⁵¹Cr) chromate (NEN).

Cells were incubated at 37°C for 1 hr with agitation.
Labelled target cells were washed 3 times with RPMI/10% FCS.

c. Setting-Up the Assays: Target cell concentration was adjusted to 10^5 /ml in RPMI/10% FCS and 100 μ l aliquots were added to each well containing responders. K562 cells (cold targets, to block NK, and LAK activity) were washed and resuspended in RPMI/10% FCS at 10^7 /ml. Aliquots of 20 μ l were added per well, yielding a 20:1 of cold K562 target:labelled target. For the determination of the spontaneous ^{51}Cr release, 100 μ l/well of RPMI/10% FCS were added to 100 μ l/well of labelled target cells, and 20 μ l/well of K562. For maximum ^{51}Cr release, 100 μ l 1% Triton X-100 (Sigma) in PBS CMF, was added to the 100 μ l/well labelled target cells, and 20 μ l/well K562. Plates were centrifuged for 2 min at 1200 rpm to accelerate cell conjugate formation. Assays were incubated for 5 hr at 37°C, 5% CO_2 . Assays were harvested by centrifuging plates for 5 min at 1200 rpm and collecting 100 μ l/well of supernatant. Standard gamma counting techniques were used to determine percent specific lysis (Micromedic automatic gamma counter, 0.5 min per tube).

Cultured Cell Lines. JY, a HLA A2.1 expressing human EBV-transformed B-cell line, was grown in RPMI/10% FCS. K562, a NK cell sensitive erythroblastoma line was grown in RPMI/10% FCS. K562 was used to reduce background killing by NK and LAK cells in the chromium release assays.

Peptides. The peptides used in these studies were synthesized at Cytel and their sequences are described in Table 24 a. Peptides were routinely diluted in 100% DMSO at 20 mg/ml, aliquoted, and stored at -20°C.

FACS Analysis. Approximately 10^6 cells were used for each antibody that was to be tested. Cells were washed twice with PBS CNU + 0.1% BSA. To each sample, 100 μ l PBS CMF + 0.1% BSA + primary antibody at 2 μ g/ml (BB7.2, ATCC) or (9.12.1, Inserm-CNRS, Marseille, France) or (LB3.1, Children's Hospital Pittsburgh) were added. A negative control was always

included. Cells were incubated on ice for 20 min and washed twice with PBS CMF + 0.1% BSA. Cells were resuspended in 100 μ l anti-mouse IgG FITC conjugate (Sigma), diluted 1:50 in PBS CMF + 0.1% BSA, and incubated 20 min on ice. Cells were washed twice with PBS CMF + 0.1% BSA, and resuspended in PBS for FACScan (Becton Dickinson) analysis. When it was necessary to postpone analysis to the subsequent days, the cells were fixed with PBS/1% paraformaldehyde (Fisher) and analyzed within one week.

Binding Assays Using Intact Cells and Radiolabelled Peptide.

JY cells were treated with citrate-phosphate buffer and neutralizing buffer #1 as described above. JY control cells were left untreated in tissue culture media. After treatment both cell populations were washed twice with serum free RPMI and loaded with 125 I-radiolabelled 941.01 (HBC15-27) peptide (standard chloramine T iodination). To determine binding specificity, 2×10^6 cells were resuspended in 200 μ l neutralizing buffer #2 (described above) containing 125 I-941.01 (10^5 cpm) \pm 100 μ g unlabelled 941.01. Cells were incubated for 4 hrs at 20°C and washed twice with serum free RPMI to remove free peptide. Cells were resuspended in 200 μ l of serum free RPMI. In a microfuge tube the cell suspension was layered over an 800 μ l FCS and pelleted by centrifugation for 5 sec. Supernatants were aspirated and the radioactivity remaining in the pellet was measured (Micromedic automatic gamma counter, 1 min per tube).

Example 15

Class I MHC molecule peptide stripping/loading by mild acid treatment.

Mild acid solutions of pH 3 such as glycine or citrate-phosphate buffers have been used by various groups to identify endogenous peptides and to identify tumor associated T cell epitopes. The treatment is unique in that only the MHC class I molecules are destabilized (and peptides released), while all other surface antigens remain intact including MHC class II molecules. Most importantly, treatment of cells with

the mild acid solutions of this example do not affect the cell's viability or metabolic state. The mild acid treatment is rapid since the stripping of endogenous peptides occurs in two minutes at 4°C and the APC is ready to perform its function after the appropriate peptides are loaded. In this example we utilized the technique to make peptide specific APCs for the generation of primary antigen-specific CTL. The resulting APC were efficient in inducing peptide-specific CD8+ CTL.

10

Measurements by FACS Analysis. PHA-induced T-cell blasts were acid stripped/peptide loaded according to the methods described in Example 15. The resulting cells were stained for FACS analysis using anti-HLA-A2 (BB7.2) and anti-HLA alpha chain-specific (9.12.1) monoclonal antibodies. Controls for this experiment included the same cell population which was not treated at pH 3 (but treated with PBS buffer at pH 7.2), and with cells treated with citrate-phosphate buffer (to strip the MHC) but neutralized in the absence of β_2 microglobulin and peptide. The results presented in Figure 15, indicate that treatment of these cells with the citrate-phosphate (pH3) buffer significantly reduced (10-fold) the reactivity of the cells toward both anti-HLA class I antibodies alone (anti-HLA-A2 and the alpha chain specific), but not towards a monoclonal antibody specific for class II MHC molecules (anti-HLA-DR). Most importantly, neutralization of the acid-stripped cells in the presence of β_2 microglobulin and peptide resulted in preservation of a significant amount of class I MHC antibody-reactive sites, with only a 2.5-fold decrease in fluorescence intensity. Importantly, the acid-treated cells remained viable, as measured by trypan blue exclusion and forward/lateral FACS scatter analysis. Similar results were obtained using EBV-transformed B cell lines, fresh (or frozen) PBMC and other peptides (which bind to either HLA-A2.1 or HLA-A1) (data not shown).

35

Binding of Radiolabeled Peptides to Empty MHC Molecules. To determine the efficiency of peptide loading using the cold

temperature incubation or acid stripping/peptide loading protocol, JY cells (an HLA-A2.1 EBV-transformed B cell line) were preincubated at 26°C overnight or acid-stripped to remove the endogenous MHC-associated peptides and the loading of exogenous peptide was determined using a ^{125}I -radiolabelled HLA-A2.1 binding peptide. The specificity of this reaction was determined by measuring the inhibition of labelled peptide binding using a cold peptide of the same sequence. Results presented in Table 24 b demonstrate that acid-treatment of the cells increased significantly (approximately 10-fold) the amount of labelled peptide binding to the JY cells. Furthermore, the binding of labelled peptide was completely blocked by the addition of the cold peptide, demonstrating specific binding (data not shown).

In Vitro Induction of Primary Antigen-Specific CTL Using Acid Stripped/Peptide Loaded APCS. Additional critical parameters for the induction of primary CTL using both the cold temperature incubation and acid strip protocol are: 1) enrichment of CD8+ T-cells in the responder cell population (or depletion of CD4+ T-cells), 2) addition of rIL-7 to the CTL induction cultures from day 0, and 3) restimulation of the cultures with antigen on day 12-14 using autologous adherent cells pulsed with peptide. Results presented in Figs. 16 and 17 show experiments performed using PBMC and PHA-induced T-cell blasts as APC. Figure 18 shows experiments using PHA-induced T-cell blasts as APC while Figure 19 shows the use of PBMC as APC.

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Example 16

Screening peptides to identify CTL epitopes.

In order to identify CTL epitopes, CTL was stimulated by SAC-I activated PBMCs as APC. Cold temperature expression of the MHC in which class I β -2-microglobulin complex is unstable was utilized in addition to acid stripping to generate PBMC APC.

Complete Culture Medium. The tissue culture medium used in this study consisted of RPMI 1640 with Hepes and L-glutamine (Gibco) supplemented with 2 mM L-glutamine (Irvine Scientific), 0.5mM sodium pyruvate (Gibco), 100 U/100 ug/ml penicillin/streptomycin (Irvine), and 5% heat-inactivated Human Serum Type AB (RPMI/5% HS; Gemini Bioproducts). Culture media used in the growth of EBV-transformed lines contained 10% heat-inactivated fetal calf serum (RPMI/10% FCS, Irvine) instead of human serum.

Cytokines. Recombinant human Interleukin-2 (rIL-2) and Interleukin-4 (rIL-4) were obtained from Sandoz and used at a final concentration of 10 U/ml and 10 ng/ml, respectively. Human interferon- γ (IFN- γ) and recombinant human Interleukin-7 (rIL-7) were obtained from Genzyme and used at 20 U/ml and 10 ng/ml, respectively.

Peptides. Peptides were synthesized at Cytel and are described in Table 24 a. Peptides were routinely diluted in 100% DMSO at 20 mg/ml, aliquoted, and stored at -70°C until use.

Cell Lines. JY, Steinlin, EHM, BVR, and KT3 are homozygous human EBV-transformed B cell lines expressing HLA A_{2.1}, A₁, A₃, A₁₁, and A₂₄, respectively. They are grown in RPMI/10% FCS. K562, an NK cell sensitive, erythoblastoma line grown in RPMI/10% FCS, was used for reduction of background killing in CTL assays. Melanoma cell lines either expressing the MAGE antigen, mel 397 and mel 938, or not expressing the MAGE antigen; mel 888, were also grown in RPMI/10% FCS.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs). Whole blood was collected into heparin containing syringes and spun in 50cc tubes at 1600 RPM (Beckman GS-6KR) for 15 minutes. The plasma layer was then removed and 10 ml of buffy coat was collected with a pipette using a circular motion. The buffy coat was mixed well and diluted with an equal volume of RPMI. The buffy coat (30 ml) was then layered on 20 ml of

Ficoll-Paque (Pharmacia) and centrifuged at 1850 RPM (400xg) for 20 minutes, 25°C, with the brake off. The interface between the Ficoll and the plasma containing the PBMCs was recovered with a transfer pipet (two interfaces per 50 ml tube) and washed three times with 50 ml of RPMI (1700, 1500, and 1300 RPM for 10 minutes). Cells were resuspended in 10-20 ml of culture medium, counted, and adjusted to the appropriate concentration.

- 10 *Freezing PBMCs.* 30 million cells/tube (90% FCS/10% DMSO; Sigma) were inserted into a Nalgene Cryo 1°C Freezing Container containing isopropanol (Fisher) and placed at -70°C from 4 hrs (minimum) to overnight (maximum). The isopropanol was changed every five times. Tubes were transferred to
- 15 liquid nitrogen for long term storage. To thaw, PBMCs were continuously shaken in a 37°C water bath until the last crystal was almost thawed (tubes were not allowed to sit in the water bath or at room temperature for any period of time). Cells were diluted into serum-free RPMI containing 30 µg/ml
- 20 DNase to prevent clumping by dead cell DNA and washed twice.

Induction of Primary CTL Using SAC-I Activated PBMCs as APCs

- a. Preparation of APCs: PBMCs were purified using the standard Ficoll-Paque protocol and resuspended at 1×10^6 /ml in RPMI/5% FCS containing 0.005% Pansorbin cells (SAC-I cells expressing Protein A; Calbiochem), 20 µg/ml Immunobeads (Rabbit anti-Human IgM; Biorad), and 20 ng/ml of human rIL-4. Two ml of cells per well were plated in a 24-well plate (Falcon, Becton Dickinson) and cultured at 37°C. After 3
- 30 days, the medium was removed and the cells were washed three times followed by addition of RPMI/10% HS. The cells were used after culturing for an additional 2 days in RPMI/10% HS.

- b. Expression of empty Class I molecules on the surface of APCs and peptide loading of APCs.
- 35

1. Cold temperature incubation:

- a. Expression of empty MHC in APCs: The APCs

were adjusted to a concentration of 2×10^6 /ml in complete culture medium containing 10 ng/ml rIL-4, 20 U/ml human IFN- γ , and 3 μ g/ml β_2 -microglobulin (β_2 m; Scripps Lab). The cells were then incubated overnight at 26°C in the presence of 5% CO₂. It should be noted that these cells only express a fraction of Class I molecules in the empty state (~10%).

b. Peptide loading of APC stimulator cells:

Empty Class I expressing APCs were washed 1-2 times with serum free RPMI (+ L-glutamine and Hepes) and resuspended at 1×10^7 in serum-free RPMI containing 50 μ g/ml total of the peptide pool (i.e., 16.7 μ g/ml of each peptide in a pool of three; 25 μ g/ml of each peptide in a pool of two; 50 μ g/ml of individual peptide), 30 μ g/ml DNase, and 3 μ g/ml β_2 m. Following a 4 hour incubation at 20°C, the cells were irradiated at 6100 rads (5×10^6 / ml; 25 million cells/tube), washed and adjusted to the appropriate concentration for addition to the induction culture (see below).

2. Acid stripping: This was used as an alternative method for generating empty MHC on the surface of the APCs. The SAC-I activated PBMCs were washed once in cold 0.9% sodium chloride (J.T. Baker) containing 1% BSA. The cells were resuspended at 10^7 /ml in cold citrate-phosphate buffer (0.13M L-ascorbic acid [J.T. Baker], 0.06M sodium phosphate monobasic [Sigma], pH3) containing 1% BSA and 3 μ g/ml β_2 m and incubated on ice. After 2 minutes, 5 volumes of cold 0.15M sodium phosphate monobasic buffer, pH7.5, containing 1% BSA, 3 μ g/ml β_2 m, and 10 μ g/ml peptide [neutralizing buffer #1] was added and the cells centrifuged at 1500 RPM for 5 minutes at 4°C. The cells were resuspended in 1 ml of cold PBS containing 1% BSA, 30 μ g/ml DNase, 3 μ g/ml β_2 -microglobulin, and 50 μ g/ml peptide [neutralizing buffer #2] and incubated for 4 hours at 20°C. As above, subsequent to the four hour incubation at 20°C, the cells were irradiated at 6100 rads (5×10^6 / ml; 25 million cells/tube), washed, then adjusted to the appropriate concentration for addition to the induction culture (see below).

c. Preparation of the CD4+ depleted PBMC responder cell population (depletion of lymphocyte sub-populations using AIS flasks). AIS MicroCollector T-150 flasks (specific for the depletion of CD4+ T cells; Menlo Park, CA) were primed by adding 25 ml of PBS/1 mM EDTA, swirling for 30 seconds so that all surfaces were moistened, and then incubating with the binding surface down at room temperature for 1 hour. Following this incubation, flasks were shaken vigorously for 30 seconds, washed 1 time with PBS/EDTA, 2 additional times with PBS and then incubated with 25 ml of culture medium for 15 minutes. PBMCs were thawed in serum-free RPMI (+ L-glutamine + Hepes) containing 30 μ g/ml DNase, washed once, and incubated for 15 minutes in culture medium. Following aspiration of culture medium from the flasks, up to 180 million PBMCs were added in 25 ml of culture medium containing 30 μ g/ml DNase. After 1 hour at room temperature, the flasks were rocked gently for 10 seconds to resuspend the nonadherent cells. The nonadherent cell suspension containing the CD8+ T cells was collected and the flasks were washed 2 times with PBS. The CD4+ T cell depleted PBMCs were centrifuged and counted for addition to the induction culture. The CD4+ and CD8+ phenotype of the CD4+ depleted cell population was determined by FACS analysis (see below). In general, this technique resulted in a two-fold enrichment for CD8+ T cells with an average of approximately 40-50% CD8+ T cells and 15-20% remaining CD4+ T cells following depletion of CD4+ T cells. Depletion of CD4+ T cells can also be accomplished by antibody and complement or antibody coated magnetic beads (Dynabeads). Depletion of CD4+ T cells served the purpose of enriching CTLp and removing cells which would compete for cell nutrients and may interfere with CTLp expansion.

d. Induction of primary CTL. During the 4 hour peptide loading of the stimulator APCs, CD4+ depleted PBMC to be used as the responder population were prepared utilizing AIS flasks for selection of CD8+ T cells through the depletion of CD4+ T cells (above). The responder cells were plated at 3×10^6 /ml in a 1 ml volume (24 well plate) and placed at 37°C until the

peptide loaded stimulator APCs were prepared. The irradiated, peptide loaded APCs were washed 1 time in serum-free RPMI (+ L-glutamine and Hepes), adjusted to 1×10^6 /ml in complete medium, and plated into a 24 well plate at 1 ml/plate: For PBMC, 1×10^6 stimulator cells (1 ml volume) were plated into the wells containing the responder cells; For SAC-I activated PBMC and PHA blasts, 1 ml of 3×10^5 /ml stimulator cells were plated in each well. A final concentration of 10 μ g/ml of additional peptide was added in addition to 10 ng/ml final concentration of rIL-7 (2 ml total volume). On day 7 an additional 10 μ g/ml rIL-7 was added to the culture and 10 U/ml rIL-2 was added every 3 days thereafter. On day 12, the cultures were restimulated with peptide pulsed adherent cells and tested for cytolytic activity 7 days later (below).

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Protocol for Restimulation of Primary CTL Using Adherent APC. PBMCs were thawed into serum-free RPMI (+ L-glutamine and Hepes) containing 30 μ g/ml DNase, washed 2 times, and adjusted to 5×10^6 /ml in culture medium containing DNase. PBMCs (25 million cells/tube in 5 ml) were irradiated at 6100R. After 1 wash, the PBMCs were resuspended in culture medium and adjusted to 4×10^6 /ml. 1 ml of irradiated PBMCs was added per well of a 24-well plate. The PBMC were incubated for 2 hours at 37°C, washed 3 times to remove non-adherent cells, and cultured in medium containing 20 μ g/ml total peptide and 3 μ g/ml β_2 microglobulin added in a 0.5 ml volume and again incubated for 2 hours at 37°C. The peptide was aspirated and 1.5×10^6 responder cells resuspended in culture medium were added in a 1 ml volume. After 2 days, 1 ml of culture medium containing 20 U/ml rIL-2 was added.

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FACS Analysis. One million cells/tube were centrifuged, resuspended in 100 μ l/tube PBS/0.1% BSA/0.02% sodium azide (Sigma) plus 10 μ l/tube directly conjugated antibody (Becton Dickinson), and incubated on ice 15-20 minutes. Cells were then washed 2 times with PBS/0.1% BSA/0.02% sodium azide and resuspended in PBS to analyze on FACScan (Beckton Dickinson). When it was not possible to analyze samples within 1-2 days,

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cells were fixed with PBS containing 1% paraformaldehyde (Fisher) and analyzed within one week.

Cytotoxicity Assay

- 5 a. Target cell preparation. Approximately 16-20 hours prior to the CTL assay, target cells (Class I matched EBV-transformed lines) were washed once and resuspended in a 10 ml volume at 3×10^5 /ml in RPMI/5% FCS in the presence or absence of 10 μ g/ml total peptide.
- 10 b. Labeling of target cells: Target cells were centrifuged and resuspended in 200 μ l/tube sodium ^{51}Cr chromate (NEN), then incubated at 37°C for 1 hour on a shaker. Targets were washed 3 times (10 ml/wash) with RPMI/10% FCS and resuspended in 10 ml (to determine the efficiency of
- 15 labelling, 50 μ l/target was counted on the Micromedic automatic gamma counter).
- 20 c. CTL assay. Target cells were adjusted to 2×10^5 /ml and 50 μ l of the cell culture was added to each well of a U-bottomed 96-well plate (Costar Corp.) for a final concentration of 1×10^4 /well. K562 cells were washed once, resuspended at 4×10^6 /ml, and 50 μ l/well was added for a final concentration of 2×10^5 /well (ratio of cold K562 to target was 20:1). Responder cells were washed once, resuspended at 9×10^6 /ml, and three fold serial dilutions
- 25 were performed for effector to target ratios of 90:1, 30:1, 10:1, and 3:1. Responder cells were added in a volume of 100 μ l in duplicate wells. For spontaneous release, 50 μ l/well of labelled target cells, 50 μ l/well K562, and 100 μ l/well of medium was added. For maximum release, 50 μ l/well target, 50 μ l/well K562, and 100 μ l/well of 0.1% Triton-X100 (Sigma) was
- 30 added. Plates were centrifuged for 5 minutes at 1200 RPM. Following a 5 hour incubation at 37°C, plates were centrifuged again for 5 minutes at 1200 RPM, and 100 μ l/well of supernatant was collected." Standard gamma counting techniques
- 35 (Micromedic automatic gamma counter; 0.5 minutes/tube) were used to determine the percent specific lysis according to the formula: % specific lysis = $\frac{\text{cpm experimental} - \text{cpm spontaneous release}}{\text{cpm maximum release} - \text{cpm spontaneous release}} \times 100$.

A cytotoxicity assay (CTL assay) was considered positive if the lysis by CTL of targets sensitized with a specific peptide at the two highest effector to target (E:T) ratios was 15% greater than lysis of control targets (i.e., target cells without peptide). A cytotoxicity assay (CTL assay) was considered borderline if the lysis by CTL of targets sensitized with a specific peptide at the two highest effector to target (E:T ratios was 6% greater than lysis of control targets (i.e., target cells without peptide).

5
10 d. Results. Of the peptides that bind to the indicated alleles, 9 of the 49 MAGE peptides, 10 of the 45 HIV peptides, 3 of the 25 HCV peptides, and 2 of the 20 HBV peptides tested to date induced primary CTL *in vitro*. Representative graphs illustrating CTL responses to various immunogenic peptides are shown for MAGE (Figure 22), HIV (Figure 23), HCV (Figure 24), and HBV (Figure 2). The CTL induction data are summarized in Table 24 which lists the immunogenic peptides which bind to the appropriate MHC and induce primary CTL *in vitro*. Indicated is the peptide's sequence, corresponding antigen and HLA allele to which it binds. Results shown in Figure 20 illustrate lysis of peptide sensitized targets and endogenous targets following stimulation with SAC-I activated PBMCs loaded with a MAGE 3 peptide, 1044.07 by the cold temperature and incubation technique. Figure 21 shows a comparison of the acid strip loading technique (Panel a) with the cold temperature incubation technique (panel b).

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30 Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

TABLE 13

VALIDATION OF CYTEL'S HLA MOTIFS

5	Binding Capacity (IC ₅₀ nM)						
	Sequence	Motif	A1	A2.1	A3.2	A11	A24
	AADKAAAAY	A1	50	--	--	--	--
	ATAKAAAAY	A1	15	--	329	77	--
	ATDKAAAAY	A1	2.8	--	9250	840	ND
10	ALAKAAAAY	A2.1	--	125	--	--	--
	AMAAAAAAK	A3.2	--	--	48	8.4	--
	ATAAAAAAK	A11	--	--	59	40	--
	AYAKAAAAY	A24	--	--	--	--	115

15 *A dash indicates an IC₅₀ greater than 20,000 nM.

TABLE 14

VALIDATION OF CYTEL'S HLA MOTIFS

Binding Capacity (IC ₅₀ nM)						
	SEQUENCE	MOTIF	A1	A2.1	A3.2	A11
	AADKAAAAAY	A1	45	--	--	--
25	ATAKAAAAAY	A1	58	--	1100	1030
	ATDKAAAAAY	A1	4.0	--	10000	4533
	ALAKAAAAAV	A2.1	ND	1400	--	--
	AMAAAAAAAK	A3.2	ND	--	85	24.0
	ATAAAAAAAK	A11	--	--	216	88

*A dash indicates an IC₅₀ greater than 20,000 nM.

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TABLE 15

HLA-A3.2

5	PEPTIDE	SEQUENCE	AVERAGE RATIO TO 952.25	SUBSTITUTION
	952.25	ALAAAAAAK	1	-
	952.26	AMAAAAAAK	1.2	position 2
	952.23	AVAAAAAAK	0.95	
	981.04	ASAAAAAAK	0.89	
10	952.24	AIAAAAAAK	0.57	
	952.27	AAAAAAAK	0.57	
	981.06	ATAAAAAK	0.49	
	981.08	AFAAAAAAK	0.13	
	981.09	AGAAAAAAK	0.077	
15	981.13	ACAAAAAAK	0.031	
	981.12	ADAAAAAAK	0.014	
	981.11	ANAAAAAAK	0.0010	
	981.05	AKAAAAAAK	<0.0016	
	981.07	AYAAAAAAK	<0.0005	
20	981.10	APAAAAAAK	<0.0006	
	952.35	ALAAAAAAR	0.46	position 9
	981.36	ALAAAAAAY	0.15	
	981.33	ALAAAAAAA	0.0034	
	981.35	ALAAAAAAQ	<0.0006	
25	981.37	ALAAAAAAS	<0.0005	
	981.38	ALAAAAAAT	<0.0005	
	981.34	ALAAAAAAN	<0.0005	
	981.39	ALAAAAAAE	<0.0003	

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TABLE 16

HLA-A11

5	PEPTIDE	SEQUENCE	AVERAGE RATIO TO 952.25	SUBSTITUTION
	952.25	ALAAAAAAK	1	-
	952.26	AMAAAAAAK	2.5	position 2
	952.27	AAAAAAAK	1.1	
	952.24	AIAAAAAAK	0.72	
10	981.06	ATAAAAAAK	0.55	
	981.04	ASAAAAAAK	0.46	
	981.09	AGAAAAAAK	0.38	
	952.23	AVAAAAAAK	0.23	
	981.11	ANAAAAAAK	0.23	
15	981.13	ACAAAAAAK	0.019	
	981.08	AFAAAAAAK	0.020	
	981.12	ADAAAAAAK	0.012	
	981.05	AKAAAAAAK	0.0065	
	981.07	AYAAAAAAK	<0.0065	
20	981.10	APAAAAAAK	<0.0051	
	952.35	ALAAAAAAR	0.015	position 9
	981.33	ALAAAAAAA	<0.0059	
	981.34	ALAAAAAAN	<0.0071	
	981.35	ALAAAAAAQ	<0.0051	
25	981.36	ALAAAAAAY	<0.0071	
	981.37	ALAAAAAAS	<0.0051	
	981.38	ALAAAAAAT	<0.0051	
	981.39	ALAAAAAAE	<0.0071	

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TABLE 17

HLA-A24

5	PEPTIDE	SEQUENCE	AVERAGE RATIO TO 983.01	SUBSTITUTION
	983.01	AYAKAAAF	1	-
	983.08	AFAKAAAF	0.24	position 2
	983.09	APAKAAAF	0.0058	
	983.10	AAAKAAAF	0.0023	
10	983.11	AKAKAAAF	<0.0012	
	983.05	AYAKAAAI	0.20	position 9
	983.04	AYAKAAAL	0.11	
	983.06	AYAKAAAV	0.0023	
	983.02	AYAKAAAA	<0.0012	
15	983.03	AYAKAAAY	<0.0012	
	983.07	AYAKAAAK	<0.0012	

TABLE 18

HLA-A1

	PEPTIDE	SEQUENCE	AVERAGE RATIO TO 982.07	SUBSTITUTION
5	982.011	ATDKAAAAAY	Motif	-
	982.07	ATAKAAAAAY	1	-
	982.09	ASAKAAAAAY	0.17	position 2
	982.13	AMAKAAAAAY	0.095	no D in pos 3
10	982.08	AAAKAAAAAY	0.0064	
	954.09	ALAKAAAAAY	0.0045	
	954.11	AIKAAAAAY	0.0045	
	954.13	AVAKAAAAAY	0.0020	
	982.10	AKAKAAAAAY	0.0011	
15	982.11	ANAKAAAAAY	<0.0001	
	982.12	ADAKAAAAAY	<0.0001	
	982.14	AGAKAAAAAY	<0.0001	
	982.15	APAKAAAAAY	<0.0001	
	982.16	AYAKAAAAAY	<0.0001	
20	982.17	AHAKAAAAAY	<0.0001	
	982.24	ATAKAAAAA	0.0040	position 9
	982.23	ATAKAAAF	0.0019	no D in pos 3
	982.28	ATAKAAAHH	0.0010	
	982.32	ATAKAAAV	0.0005	
25	982.25	ATAKAAAN	<0.0001	
	982.26	ATAKAAAD	<0.0001	
	982.27	ATAKAAAW	<0.0001	
	982.30	ATAKAAAK	<0.0001	
	982.31	ATAKAAAI	<0.0001	
30	982.29	ATAKAAAP	<0.0001	

TABLE 19

HLA-A1

5	PEPTIDE	SEQUENCE	AVERAGE RATIO TO 982.07	SUBSTITUTION
	982.01	ATDKAAAAY	Motif	-
	982.07	ATAKAAAAY	1	-
10	982.01	AADKAAAAY	0.14	position 3
	954.03	AAEKAAAAY	0.038	no T in pos 2
	982.02	AAAKAAAAY	0.0055	
	982.06	AASKAAAAY	0.0024	
	982.04	AANKAAAAY	0.0011	
	982.03	AAQKAAAAY	0.0008	
	982.05	AAKKAAAAY	<0.0001	
15	982.20	AADKAAAAA	0.0016	position 9
	982.21	AADKAAAAY	0.0005	no T in pos 2
	982.19	AADKAAAAY	<0.0001	
	982.22	AADKAAAAY	<0.0001	
	982.22	AADKAAAAY	<0.0001	

TABLE 20 (A)

HPV16 E6 AND E7 PEPTIDES BINDING TO HLA-A1

5					
	Origin	First aa Position	Sequence [*]	Binding Ratio to Standard [†]	Motif Prediction
	E6	80	ISEYRHYAY	3.500	+
	E6	69	VADKALKFY	0.240	+
	E7	44	QAEPDRAHY	0.029	+
10	E7	37	EIDGPAGQA	0.025	-
	E7	19	TTDLYAYEQ	0.023	+
	E6	144	MSAARSSRT	0.019	+/-
	E7	73	HVDIRTLED	0.014	-
	E6	139	WTGRAMSAA	0.010	-
15	E6	61	YRDGNPYAV	0.008	-

* Bold A's indicate residues in which cysteine was replaced by alanine.

† The average IC₅₀ value \pm SE of the standard in the course of the experiments considered in this table was 81 \pm 30 nM. Listed in the table are peptides yielding ratio values of \geq 0.001. All other peptides yielded ratio values of \leq 0.001.

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TABLE 20(B)
HPV16 E6 and E7 Peptides Binding to HLA-A3.2

5	Origin	First aa Position	Sequence*	Binding Ratio to Standard†	Motif Prediction
	E6	107	LIRAINAQK	3.7000	+
	E6	59	IVYRDGNPY	3.0000	+
	E7	89	IVAPIASQK	2.2000	+
10	E6	33	IILEAVYAK	1.5000	+
	E6	125	HLDKKQRFH	0.4400	+
	E6	143	AMSAARSSR	0.1800	+
	E6	7	AMFQDPQER	0.1000	+
	E6	93	TTLEQQYNK	0.0780	+
15	E6	37	AVYAKQQLL	0.0320	-
	E7	51	HYNIVTFAA	0.0210	-
	E6	145	SAARSSRTR	0.0200	+
	E6	75	KFYSKISEY	0.0100	+
	E6	89	SLYGTILEQ	0.0080	-
20	E7	52	YNIVTFAAK	0.0067	-
	E6	80	ISEYRHYAY	0.0064	+
	E6	42	QQLLRREVY	0.0058	-
	E6	68	AVADKALKF	0.0056	+
	E6	97	QQYNKPLAD	0.0045	-
25	E6	79	KISEYRHYA	0.0044	-
	E6	84	RHYAYSLYG	0.0036	-
	E6	69	VADKALKFY	0.0025	+
	E6	146	AARSSRTRR	0.0020	+
	E7	58	AAKADSTLR	0.0016	+
30	E6	38	VYAKQQLLR	0.0012	-
	E6	67	YAVADKALK	0.0012	+
	E7	60	KADSTLRLA	0.0012	-

* Bold A's indicate residues in which cysteine was replaced by alanine.

† The average IC₅₀ value ±SE of the standard in the course of the experiments considered in this table was 30±3 nM. Listed in the table are peptides yielding ratio value of ≥0.001. All other peptides yielded ratio values of <0.001.

Table 20(C)
HPV16 E6 and E7 Peptides Binding to HLA-A11.2

5	Origin	First aa Position	Sequence*	Binding Ratio to Standard†	Motif Prediction
	E6	33	IILEAVYAK	6.7000	+
	E6	93	TTLEQQYNK	1.8000	+
	E7	89	IVAPIASQK	1.3000	+
10	E6	7	AMFQDPQER	0.8400	+/-
	E6	59	IVYRDGNPY	0.4700	- (+)§
	E6	80	ISEYRHYAY	0.4300	- (+)§
	E6	37	AVYAKQQLL	0.0450	-
	E6	145	SAARSSRTR	0.0330	+/-
15	E6	107	LIRAINAQK	0.0120	+
	E7	58	AAKADSTLR	0.0110	+/-
	E6	42	QQLLRREYV	0.0084	+/- (+)§
	E6	143	AMSAARSSR	0.0084	-
	E6	79	KISEYRHYA	0.0076	-
20	E6	67	YAVADKALK	0.0074	+
	E7	52	YNIVTFAAK	0.0060	+
	E6	68	AVADKALKF	0.0037	-
	E6	69	VADKALKFY	0.0030	- (+)§
	E6	38	VYAKQQLLR	0.0022	+/-
25	E6	140	TGRAMSAAR	0.0012	+/-
	E7	90	VAPIASQKP	0.0012	-
	E7	51	HYNIVTFAA	0.0010	-

- * Bold A's indicate residues in which cysteine was replaced by alanine.
† The average IC₅₀ value ±SE of the standard in the course of the experiments considered in this table was 10±3 nM. Listed in the table are peptides yielding ratio value of ≥0.001. All other peptides yielded ratio values of <0.001.
§ Brackets indicate score according to adjusted motif.

Table 20 (D)

HPV16 E6 and E7 Peptides Binding to HLA-A24

5	Origin	First aa Position	Sequence*	Binding Ratio to Standard†	Motif Prediction
	E6	87	AYS LY GTTL	0.1200	+
	E6	72	KALKFYSKI	0.1100	- (+)‡
	E6	131	RFHNIRGRW	0.1000	+
10	E7	49	RAHYNIVTF	0.0670	- (+)‡
	E6	49	VYDFAFRDL	0.0610	+
	E6	82	EYRHYAYSL	0.0460	+
	E6	26	LQTTIHDI	0.0200	-
	E6	66	PYAVADKAL	0.0055	-
15	E6	1	MHQKRTAMF	0.0049	-
	E6	85	HYAYS LY GT	0.0037	-
	E6	44	LLRRE VY DF	0.0023	+
	E6	38	VYAKQOLLR	0.0011	-

- * Bold A's indicate residues in which cysteine was replaced by alanine.
- † The average IC₅₀ value ±SE of the standard in the course of the experiments considered in this table was 22±6 nM. Listed in the table are peptides yielding ratio value of ≥0.001. All other peptides yielded ratio values of <0.001.
- ‡ Brackets indicate score according to adjusted motif.

TABLE 20 (B)
SUMMARY OF EFFICACY OF 2, (3), 9 ANCHOR MOTIFS

Percentage Binders with Motifs	HLA Allele			
	A1	A3.2	A11.2	A24
	Predicted/Found	Predicted/Found	Predicted/Found	Predicted/Found
High (≥ 0.1)	2 2(100%)	7 7(100%)	6 6(100%)	2 3(67%)
Intermediate (0.1-0.01)	3 6(50%)	3 5(60%)	3 4(75%)	2 4(50%)
Weak (0.01-0.001)	0 1(0%)	6 14(43%)	6 11(55%)	1 5(20%)
Negative (≤ 0.001)	7 231(3%)	16 214(7%)	14 219(6%)	2 228(1%)
Totals	12 240	32 240	26 240	7 240

TABLE 21 (A)
A3.2 9-MER OPTIMAL MOTIFS

9-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
IK	3(15%)	4(20%)	7(35%)	6(30%)	20(100%)
IR	3(15.8%)	4(21.1%)	7(36.8%)	5(26.3%)	19(100%)
LK	13(48.1%)	6(22.2%)	5(18.5%)	3(11.1%)	27(100%)
LR	4(8.5%)	9(19.1%)	20(42.6%)	14(29.8%)	47(100%)
SK	0	0	0	0	0
SR	0	0	0	1(100%)	1(100%)
TK	3(15%)	6(30%)	7(35%)	4(20%)	20(100%)
TR	2(16.7%)	1(6.0%)	2(16.7%)	7(58.3%)	12(100%)
VK	9(28.1%)	13(40.6%)	9(28.1%)	1(3.1%)	32(100%)
VR	2(7.4%)	3(11.1%)	14(51.9%)	8(29.6%)	27(100%)
Total	39(19%)	46(22.4%)	71(34.6%)	49(23.9%)	205(100%)

TABLE 21 (B)
ALL 9-MER OPTIMAL MOTIFS

9-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
GK	0	1 (100%)	0	0	1 (100%)
IK	5 (25%)	5 (25%)	7 (35%)	3 (15%)	20 (100%)
LK	6 (22.2%)	10 (37%)	9 (33.3%)	2 (7.4%)	27 (100%)
TK	10 (50%)	4 (20%)	4 (20%)	2 (10%)	20 (100%)
VK	12 (37.5%)	15 (46.9%)	4 (12.5%)	1 (3.1%)	32 (100%)
Total	33 (33%)	35 (35%)	24 (24%)	8 (8%)	100 (100%)

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TABLE 22(A)
A3.2 10-MER OPTIMAL MOTIFS

10-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
AK	1(33.3%)	1(33.3%)	1(33.3%)	0	3(100%)
AR	0	0	1(100%)	0	1(100%)
FR	0	0	0	0	0
IK	0	0	1(25%)	3(75%)	4(100%)
IR	1(17.1%)	6(27.3%)	10(45.5%)	6(27.3%)	22(100%)
LK	16(53.3%)	1(7.1%)	2(14.2%)	10(71.4%)	30(100%)
LR	4(12.5%)	7(23.3%)	5(16.7%)	2(6.7%)	32(100%)
MK	1(100%)	9(28.1%)	11(34.3%)	8(25%)	32(100%)
MR	1(100%)	0	0	0	1(100%)
TK	2(11.8%)	5(29.4%)	8(47.1%)	2(11.8%)	17(100%)
TR	1(4.8%)	1(4.8%)	9(42.9%)	10(47.6%)	21(100%)
VK	7(35%)	4(20%)	5(25%)	4(20%)	20(100%)
VR	0	6(21.4%)	15(53.6%)	7(25%)	28(100%)
Total	34(117.5%)	40(120.6%)	68(135.1%)	52(126.8%)	194(100%)

TABLE 22 (B)
All 10-MER OPTIMAL MOTIFS

10-mers	GOOD BINDERS	INTERMEDIATE BINDERS	WEAK BINDERS	NON-BINDERS	TOTAL
AK	1 (33.3%)	1 (33.3%)	1 (33.3%)	0	3 (100%)
CK	0	0	1 (100%)	0	1 (100%)
GK	0	0	0	0	0
IK	4 (18.2%)	5 (22.7%)	12 (54.5%)	1 (4.5%)	22 (100%)
LK	9 (30%)	12 (40%)	8 (26.7%)	1 (3.3%)	30 (100%)
MK	1 (100%)	0	0	0	1 (100%)
TK	6 (35.3%)	5 (29.4%)	5 (29.4%)	1 (5.9%)	17 (100%)
VK	7 (35%)	8 (40%)	4 (20%)	1 (5%)	20 (100%)
Total	28 (29.8%)	31 (33%)	31 (33%)	4 (4.3%)	94 (100%)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0300	HLDMLRHLY	9	c-EBR2			42	1	9.1	0.007	0.002	
1.0304	LLDIDETRY	9	c-EBR2			89	1	7.6	0.003	0	
1.0305	GTQLFEDNY	9	c-EBR2			104	1	0.18	0	0.029	
1.0305	LTCSPQFEY	9	c-EBR2			1131	1	0.13	0	0.0061	
1.0317	ETLEBITCY	9	c-EBR2			401	1	0.043			
1.0749	PTHQSDVWSY	10	c-EBR2			899	1	2.7	0.0008	0.0025	
1.0747	RLLDIDETRY	10	c-EBR2			848	1	1.3	0.0017	0	
1.0715	TLEBITCYLY	10	c-EBR2			402	1	1.1	0	0	
1.0737	YVMAGVCSPY	10	c-EBR2			772	1	1.1	0.010	0.012	0
1.0744	GTPTAENPEY	10	c-EBR2			1239	1	0.063			
1.0706	LQRPQQLCY	10	c-EBR2			154	1	0.000			
1.0693	VVQGNLELY	10	c-EBR2			55	1	0.018			
1.0754	MCDLVDAEY	10	c-EBR2			1014	1	0.012			
1.1028	KRKTYMR	9	c-EBR2			681	3.11		0.76	0.0018	
1.1027	VVFCILIK	9	c-EBR2			649	3.11		0.11	0.72	
1.0344	LVKSPNHYK	9	c-EBR2			852	3.11		0.48	0.070	
1.0305	VLKENTSPK	9	c-EBR2			754	3.11		0.40	0.013	
1.0329	RLKRNQCK	9	c-EBR2			673	3.11		0.36	0.0097	
1.0311	ILWKDQPHK	9	c-EBR2			167	3.11		0.28	0.31	
1.1003	KITDCLAR	9	c-EBR2			860	3.11		0.17	0.34	
1.0849	GVVFCILIK	9	c-EBR2			648	3.11		0.0047	0.089	
1.0299	QVCTGIDMK	9	c-EBR2			24	3.11		0.0007	0.002	
1.1001	LLDHVRENK	9	c-EBR2			805	3.11		0.007	<0.0004	
1.1026	CVNCSQRL	9	c-EBR2			538	3.11		0.0015	0.001	
1.1023	TVCAGCCAR	9	c-EBR2			218	3.11		0.0004	0.003	
1.0331	ILKETELK	9	c-EBR2			714	3.11		0.019	0.0023	
1.1024	VTAEDGTOR	9	c-EBR2			322	3.11		<0.0002	0.014	
1.0306	DLSPPIWK	9	c-EBR2			607	3.11		0.0005	0.008	
1.0707	TILWKDQPHK	10	c-EBR2			166	3.11		0.043	3.4	
1.0712	GTQCEKCKK	10	c-EBR2			307	3.11		0.021	0.61	
1.0734	KVLKENTSPK	10	c-EBR2			753	3.11		0.36	0.22	
1.0702	QLSLTELK	10	c-EBR2			143	3.11		0.30	0.013	
1.1142	RLVHRLAAR	10	c-EBR2			840	3.11		0.18	0	
1.0741	LLNWCQIAK	10	c-EBR2			822	3.11		0.14	0.34	
1.0732	TIDVYMBVK	10	c-EBR2			948	3.11		0.023	0.12	
1.0731	RLKETELK	10	c-EBR2			713	3.11		0.057	0.11	
1.0745	VLKSPNHYK	10	c-EBR2			851	3.11		0.002	0.0072	
1.1131	SVFQNLQVIR	10	c-EBR2			423	3.11		0.017	0.075	
1.1133	HTVPWDQLR	10	c-EBR2			476	3.11		0.0005	0.071	
1.1127	ILKGCVLQR	10	c-EBR2			148	3.11		0.040	0.0005	
1.1143	LVSESDMAR	10	c-EBR2			972	3.11		0.0072	0.003	
1.1136	GVVFCILIK	10	c-EBR2			648	3.11		0.018	0.003	
1.0726	CVARCPSVK	10	c-EBR2			596	3.11		0.027	0.0042	
1.1137	VVFCILIKR	10	c-EBR2			649	3.11		0.0030	0.016	
1.0728	GLKRNQCK	10	c-EBR2			672	3.11		0.015	0.0014	
1.1129	RTVCAGCCAR	10	c-EBR2			217	3.11		0.0048	0.013	
1.1134	GLACIQCAR	10	c-EBR2			508	3.11		0.011	0	
1.1139	KPFVARKLR	10	c-EBR2			747	3.11		0.0009	0.0099	

Table 23(a)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0291	VCEADYFEY	9	EBNA1			409	1	0.016			
1.0295	PLRESIVCY	9	EBNA1			553	1	0.010			
1.0451	IVCEADYFEY	10	EBNA1			408	1	0.013			
1.0483	GTWVACVPVY	10	EBNA1			501	1	0.014			
1.0283	GVFVYGCSK	9	EBNA1			506	3.11		0.30	0.61	
1.1016	KTSLYNLER	9	EBNA1			514	3.11		0.31	0.12	
1.0297	AKDLVMTK	9	EBNA1			578	3.11		0.048	0.004	
1.0687	QTHFAEVLK	10	EBNA1			567	3.11		0.010	0.21	
1.1124	GTALAIFQCR	10	EBNA1			523	3.11		0.0028	0.056	

Table 23(b)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A32	A11	A24
S.0005	CTELKLSOY	9	FLU	A	NP	44	1	3.6			
S.0008	STLELSRY	9	FLU	A	NP	377	1	0.020			
S.0044	ILRGSAHK	9	FLU	A	NP	245	3		1.5	0.0037	
S.0051	RMGNILKCK	9	FLU	A	NP	221	3		0.27	0.062	
S.0046	LMQGSTLPK	9	FLU	A	NP	146	3		0.031	0.10	
S.0048	MIDGICRPY	9	FLU	A	NP	32	3		0.059	0.0010	
S.0049	MVLSAFDER	9	FLU	A	NP	66	3		0.0016	0.041	
S.0054	YIQMCTELK	9	FLU	A	NP	40	3		0.0003	0.000	
S.0062	GNDRNFWK	9	FLU	A	NP	200	3		0.0028	0.024	
S.0104	SLMQGSTLPK	10	FLU	A	NP	165	3		0.12	0.84	
S.0095	KMDGICRPY	10	FLU	A	NP	31	3		0.30	0.0079	
S.0096	LILRGSAHK	10	FLU	A	NP	244	3		0.36	0.037	
S.0102	RSCAACAANK	10	FLU	A	NP	175	3		0.019	0.0046	
S.0105	SSTLELSRY	10	FLU	A	NP	376	3		0.0018	0.016	
S.0103	RSRYWAIRTK	10	FLU	A	NP	382	3		0.012	0	
S.0101	RMVLSAFDER	10	FLU	A	NP	65	3		0.0014	0.010	
S.0061	PYQMCTEL	9	FLU	A	NP	39	24				2.9
S.0060	AYERMCTEL	9	FLU	A	NP	218	24				0.031
S.0112	RFYQMCTEL	10	FLU	A	NP	38	24				0.15

Table 23(c)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0153	LLDTASALY	9	HBV	adr	CORE	420	1	25	0.0007	0	
1.0186	SLDVSAAPY	9	HBV	adr	POL	1001	1	17.2	0.0007	0.0006	
2.0125	PTTGKTSLY	9	HBV	ALL		1362	1	1.3	0.0009	0	
2.0126	MSTTDLEAY	9	HBV	adr		1521	1	0.85	<0.0008	0	
1.0208	PTTGKTSLY	9	HBV	adr	POL	1362	1	0.77	0	0	
1.0387	LTKQYLNLV	9	HBV	adw	POL	1280	1	0.50	0.0003	0.0075	
2.0122	LTKQYLNLV	9	HBV	adw		1280	1	0.095			
1.0166	KVGNPTGLY	9	HBV	adr	POL	629	1	0.048			
2.0127	MSPTDLEAY	9	HBV	adw		1550	1	0.067			
2.0120	PSQPSQNY	9	HBV	ayw		984	1	0.057			
2.0112	PSWAFAYK	9	HBV	adw		316	1	0.054			
2.0119	QSAVRKEAY	9	HBV	adw		881	1	0.025			
1.0174	FLDKGIGPY	9	HBV	adr	POL	698	1	0.019			
1.0378	SLMLLYKTY	9	HBV	adw	POL	1092	1	0.017			
2.0115	ASRDLVVS	9	HBV	ayw		499	1	0.013			
2.0124	PSRRLGLY	9	HBV	adr/adw		1364	1	0.011			
2.0121	SSTSRNRY	9	HBV	adr		1036	1	0.0097			
1.0519	DLDTASALY	10	HBV	adr	CORE	419	1	11.1	0	0	
1.0513	LLDPVYGLY	10	HBV	adr	ENV	120	1	6.3	0.17	0	
2.0239	LSLDSAAFY	10	HBV	ALL		1000	1	4.2	<0.0009	0.0007	
1.0911	FLQQYLNLV	10	HBV	adr	POL	1280	1	1.1	0.014	0.0048	0.0017
2.0216	QTFCRGLHY	10	HBV	ayw	POL	1087	1	1.1	0.0056	0.002	
2.0244	KTYCRGLHY	10	HBV	adw		1088	1	0.69	0.59	0.22	0
1.0991	KTYCRGLHY	10	HBV	adw	POL	1088	1	0.57	0.53	0.36	0.0001
2.0242	QTFCRGLHY	10	HBV	ayw		1087	1	0.37	0.0037	0.011	
1.0554	KTFGRGLHY	10	HBV	adr	POL	1069	1	0.34	0.094	0.090	0
2.0241	KTFGRGLHY	10	HBV	adr		1069	1	0.30	0.15	0.095	0
1.0966	LQDPVYRALY	10	HBV	adw	ENV	120	1	0.21	0.014	0	
1.0806	TTPAQGTSMY	10	HBV	adw	ENV	288	1	0.20	0	0	
2.0240	LSSTSRNRY	10	HBV	adr		1035	1	0.20	<0.0009	0	
1.0541	FLDKGIGPY	10	HBV	adr	POL	698	1	0.16	0	0	
2.0238	HSASPCSPY	10	HBV	ayw		767	1	0.15	0.019	0.017	0
1.0995	FLTKQYLNLV	10	HBV	adw	POL	1279	1	0.12	0	0	
2.0237	HSASPCSPY	10	HBV	adr/adw		738	1	0.11	0.003	0.020	0
1.0774	WLWQMDIDFY	10	HBV	adw	CORE	416	1	0.081			
2.0233	TTPAQGTSMY	10	HBV	ayw		288	1	0.046			
1.0542	HTLWKAGLY	10	HBV	adr	POL	723	1	0.030			
2.0231	TSQFPICGY	10	HBV	adr		226	1	0.018			
2.0246	KSVQHLESY	10	HBV	adw		1361	1	0.016			
1.0910	NLYVSLLLY	10	HBV	adr	POL	1089	1	0.015			
1.0901	WMWVWYWCPL	10	HBV	adr	ENV	359	2.1	0.0009	0.019	0	0.0005
2.0089	LLYQTGRK	9	HBV	ayw	POL	1084	3	1.8	0.64		
2.0116	IMPARFPYK	9	HBV	ayw		713	3	0.99	1.5		
2.0082	CLHQSPVRK	9	HBV	ayw	POL	867	3	0.14	0.025		
5.0056	SAKSVVRR	9	HBV		POL	531	3	<0.0003	0.067		
2.0077	HLHQDIKK	9	HBV	ayw	POL	686	3	0.041	0.0095		
2.0219	SLPQEHQK	10	HBV	ayw	POL	1197	3	0.36	4.2		
2.0234	SMFPSCCTK	10	HBV	adr/adw		295	3	0.43	1.9		
2.0235	SMFPSCCTK	10	HBV	ayw		295	3	1.1	1.79		
5.0107	QAFTSPITYK	10	HBV		POL	446	3	0.15	1.3		
2.0214	LLYQTGRK	10	HBV	ayw	POL	1083	3	0.89	0.021		
2.0245	YMDQVVLCAK	10	HBV	ALL		1123	3	0.16	0.0096		
5.0108	TSKSVVRR	10	HBV		POL	530	3	0.0006	0.013		
2.0094	PTYKARLCK	9	HBV	ayw	POL	1243	11	0.080	0.085		
2.0048	PTDLEAYFK	9	HBV	adw	"	1582	11	0.0002	0.016		
2.0061	KYTSFPWLL	9	HBV	ALL		1330	24				3.6
2.0069	LYAAVTNRL	9	HBV	adw		1169	24				3.2
2.0046	FYPNLTLYL	9	HBV	adr		689	24				2.1
2.0045	LYSSTVPSF	9	HBV	adw/ayw		685	24				1.9
2.0048	FYPKVTLYL	9	HBV	ayw		718	24				1.7
2.0049	FYPNVTLYL	9	HBV	adw		718	24				1.6
2.0039	LYSLSPFL	9	HBV	ayw		368	24				0.50
2.0044	LYSSTVPVL	9	HBV	adr		636	24				0.37
2.0038	LYNLSFPL	9	HBV	adr		368	24				0.34
2.0051	NYRVSVPKF	9	HBV	ayw		991	24				0.18
2.0050	HYQTRHYL	9	HBV	adw/ayw		743	24				0.15
2.0047	HYFKTRHYL	9	HBV	adr		714	24				0.057
2.0060	GYPALMPLY	9	HBV	ALL		1224	24				0.049
5.0062	AYKPTNAP	9	HBV		NUC:NUC:PUS	131	24				0.026
2.0054	LYQTGRKL	9	HBV	ayw		1085	24				0.014
2.0043	SYQHFRLL	9	HBV	ayw		607	24				0.011
2.0181	LYSHPLGF	10	HBV	ALL		1077	24				1.1
2.0182	LYAAVTNPL	10	HBV	adw		1169	24				0.32
2.0188	LYRPLSLPF	10	HBV	adr		1371	24				0.25

Table 23(d)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
2.0174	SYQHPRIILL	10	HBV	ayw		607	24				0.16
2.0173	SYQHPRIKILL	10	HBV	adr/adw		578	24				0.066
2.0176	YYPEHLVNIY	10	HBV	ayw		735	24				0.040
2.0172	AYRPPNAPIL	10	HBV	ALL		521	24				0.022
2.0171	CYKWMCLRRF	10	HBV	ALL		234	24				0.011
3.0115	NRLSLGHL	10	HBV		FOL	572	24				0.0099
1.0377	YVSLMLLYK	9	HBV	adw	FOL	1080	3.11		0.31	7.4	
1.0189	LLYKTYGRK	9	HBV	adr	FOL	1066	3.11		5.0	0.30	
1.0379	LLYKTYGRK	9	HBV	adw	FOL	1095	3.11		2.5	0.40	
1.0370	VTKYLPLOK	9	HBV	adw	FOL	722	3.11		0.014	1.3	
1.0176	RHYLHTLWK	9	HBV	adr	FOL	719	3.11		1.2	0.010	
1.0367	STVPSNPK	9	HBV	adw	FOL	668	3.11		0.021	0.93	
1.0215	TIDLEAYPK	9	HBV	adr	X	1523	3.11		0.0006	0.92	
1.0848	YVSLLLLYK	9	HBV	adr	FOL	1061	3.11		0.39	0.92	
1.0383	PTYKAPLYK	9	HBV	adw	FOL	1274	3.11		0.17	0.71	
1.0867	HLVYVARQR	9	HBV	adr	FOL	1257	3.11		0.34	0.0020	
1.0368	STNRQLCEK	9	HBV	adw	ENV	85	3.11		0.51	0.34	
1.0971	ALRPTSAKR	9	HBV	adr	X	1488	3.11		0.44	<0.0005	
1.0197	PVNSPDWKE	9	HBV	adr	FOL	1197	3.11		0.080	0.41	
1.0369	TVNBNRLK	9	HBV	adw	FOL	703	3.11		0.064	0.40	
1.1041	VVNSYQTR	9	HBV	adw	FOL	740	3.11		0.080	0.38	
1.0152	STSRGCK	9	HBV	adr	ENV	277	3.11		0.011	0.29	
1.0213	QVLPKLLHK	9	HBV	adr	X	1505	3.11		0.10	0.28	
1.0172	UTKYLPLOK	9	HBV	adr	FOL	683	3.11		0.0009	0.23	
1.0274	CLHQSAVRK	9	HBV	adw	FOL	878	3.11		0.22	0.017	
1.0860	VVDSPQSR	9	HBV	adr	FOL	943	3.11		0.011	0.20	
1.0382	FLYACQAK	9	HBV	adw	FOL	1259	3.11		0.18	0.084	
2.0274	YVNTNMLK	9	HBV	ayw	CORE	507	3.11		0.16	0.048	
1.0199	FLYACQSK	9	HBV	adr	FOL	1230	3.11		0.11	0.018	
1.0972	RLADEGLNR	9	HBV	adr	FOL	601	3.11		0.10	0.025	
1.0876	AVNHYFKTR	9	HBV	adr	FOL	711	3.11		0.0071	0.098	
1.0875	RLKLMFAR	9	HBV	adr	FOL	680	3.11		0.085	0.0002	
1.0977	RLYKRIYTK	9	HBV	adr	FOL	730	3.11		0.085	<0.0005	
1.0993	KVPVLGCCR	9	HBV	adr	X	1548	3.11		0.002	0.082	
1.0145	NVSPWTHK	9	HBV	adr	FOL	631	3.11		0.072	0.094	
1.0882	LLLYKTGR	9	HBV	adr	FOL	1055	3.11		0.072	0.0045	
1.0978	RLVQYSTR	9	HBV	adr	FOL	757	3.11		0.064	0.0082	
1.0219	PVLGCCRHK	9	HBV	adr	X	1580	3.11		0.065	0.019	
1.1042	RLVQYSTR	9	HBV	adw	FOL	785	3.11		0.064	0.0002	
1.1043	MLLYKTYGR	9	HBV	adw	FOL	1094	3.11		0.061	0.0032	
1.0170	TVNBNRLK	9	HBV	adr	FOL	674	3.11		0.048	0.007	
1.1045	NLYPVARQR	9	HBV	adw	FOL	1286	3.11		0.042	0.0011	
1.1046	LPVRYPTGR	9	HBV	adw	FOL	1407	3.11		0.021	0	
1.0845	LVSPGVYMR	9	HBV	adr	CORE	509	3.11		0.0003	0.020	
1.0981	LVGSCLPK	9	HBV	adr	FOL	1022	3.11		0.0008	0.015	
1.0867	HSCLTYGR	9	HBV	adr	CORE	494	3.11		0.013	0.011	
1.1047	SVPSLFDK	9	HBV	adw	FOL	1404	3.11		0.0007	0.010	
1.0889	SVPSLFDK	9	HBV	adr	FOL	1395	3.11		0.0004	0.010	
1.0844	TLVQSRVLK	10	HBV	adr	FOL	1179	3.11		0.092	5.6	
2.0205	TVFVNRHWK	10	HBV	ayw	FOL	640	3.11		0.0007	4.2	
1.0843	TLWKAGLYK	10	HBV	adr	FOL	724	3.11		3.5	1.0	
1.0807	SMVPSQCTK	10	HBV	ayw	ENV	285	3.11		1.5	1.4	
1.1153	RLPYRYTKR	10	HBV	adw	FOL	1406	3.11		2.8	0.080	
1.0884	STIDLEAYPK	10	HBV	adr	X	1522	3.11		0.0046	2.7	
1.0854	LLLYKTGRK	10	HBV	adr	FOL	1065	3.11		2.5	0.012	
1.0799	TVNAHRLPK	10	HBV	adw	X	1529	3.11		0.007	0.45	
1.0886	EAYPKDCLK	10	HBV	adr	X	1527	3.11		0.007	0.74	
1.1081	LVVDSPQSR	10	HBV	adr	FOL	942	3.11		0.0009	0.63	
1.0789	MLLYKTYGRK	10	HBV	adw	FOL	1094	3.11		0.61	0.020	
1.0846	TAYSHLTSK	10	HBV	adr	FOL	858	3.11		0.26	0.092	
1.0842	SLGHLNPNK	10	HBV	adr	FOL	1150	3.11		0.20	0.078	
1.1152	RLGLYPLLR	10	HBV	adw	FOL	1397	3.11		0.19	0.0049	
1.0847	VTCGVFLVDK	10	HBV	adr	FOL	943	3.11		0.085	0.17	
1.1150	RRTPKTPAR	10	HBV	adw	FOL	942	3.11		0.17	0.0002	
1.0881	TVNGHQVLPK	10	HBV	adr	X	1500	3.11		0.073	0.092	
1.1091	SLPQPTTKR	10	HBV	adr	FOL	1377	3.11		0.077	0.043	
1.1072	TLPETTVYR	10	HBV	adr	CORE	532	3.11		<0.0003	0.075	
1.1089	CTDNSVLSR	10	HBV	adr	FOL	1320	3.11		0.025	0.072	
1.1071	SLPETTVYR	10	HBV	adr	CORE	531	3.11		0.0005	0.068	
2.0210	KVTKYLPLOK	10	HBV	ayw	FOL	721	3.11		0.027	0.083	
1.1148	STRHCDKSR	10	HBV	adw	FOL	792	3.11		0.0057	0.038	
1.0935	VLSGWLOFR	10	HBV	adw	FOL	923	3.11		0.029	0.0087	
1.0781	NVTKYLPLOK	10	HBV	adw	FOL	721	3.11		<0.0004	0.023	

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A1.2	A11	A24
1.1092	RVCCQLDPAR	10	HBV	adr	X	1422	3.11		0.0019	0.023	
1.0793	SLGIHLNPOK	10	HBV	adw	POL	1179	3.11		0.017	0.014	
1.0909	YLVSRQVWIR	10	HBV	adr	CORE	508	3.11		0.015	0.0027	
2.0207	FVCPLTVNEK	10	HBV	ayw	POL	698	3.11		0.0057	0.015	
1.0535	YVCPLTVNEK	10	HBV	adr	POL	649	3.11		0.0049	0.014	
1.1075	RLADEGLNKK	10	HBV	adr	POL	601	3.11		0.013	0.0004	
1.1086	IVLKLQCFK	10	HBV	adr	POL	1185	3.11		0.013	0.0024	
1.0773	PFSWAFK	10	HBV	adw	ENV	314	3.11		<0.0003	0.010	
1.0778	LTVNENRRLK	10	HBV	adw	POL	702	3.11		0.0025	0.0095	

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Metif	A1	A3.2	A11	A24
1.0118	CTCGSSDLY	9	HCV		LORF	1123	1	3.0	0	0.010	
1.0112	NIVDVQVLY	9	HCV		NS1/ENV2	697	1	0.60	0	0.010	
2.0034	VQDCNCSY	9	HCV			302	1	0.54	0.0005	0.0003	
2.0035	LTPROMVDY	9	HCV			605	1	0.078			
1.0145	RVCEOMALY	9	HCV		LORF	2588	1	0.053			
1.0140	DVCCSMYS	9	HCV		LORF	2416	1	0.039			
2.0036	PTIRKIMY	9	HCV			636	1	0.012			
1.0509	GLSAPSLHSY	10	HCV		LORF	2888	1	0.41	0.013	0.0034	0.0002
1.0489	TLHCFTPLY	10	HCV		LORF	1617	1	0.30	0.11	0.0026	
2.0037	EYVLLFL	9	HCV			719	24				1.4
2.0169	MYVCGVEHRL	10	HCV			633	24				0.026
2.0170	EYVLLFL	10	HCV			719	24				0.010
1.0139	SVPABLRK	9	HCV		LORF	2269	3.11		0.016	0.87	
1.0855	QLFTSPRR	9	HCV		ENV1	290	3.11		0.75	0.083	
1.0090	RLGVKATRK	9	HCV		CORE	43	3.11		0.74	0.16	
1.0123	LIFCHSKK	9	HCV		LORF	1391	3.11		0.54	0.19	
1.0122	HLFCHSKK	9	HCV		LORF	1390	3.11		0.25	0.010	
1.0852	KTSERSQPR	9	HCV		CORE	51	3.11		0.16	0.064	
1.0120	AVCTRGVAK	9	HCV		LORF	1183	3.11		0.016	0.088	
1.0143	EVFCVQFEK	9	HCV		LORF	2543	3.11		0.0019	0.083	
1.0137	ITRVSENK	9	HCV		LORF	2241	3.11		0.015	0.0099	
1.0857	CHTSLTGR	9	HCV		LORF	1042	3.11		0.0085	0.011	
1.0496	GVAGALVAFK	10	HCV		LORF	1858	3.11		0.87	1.1	
1.0480	HLHAPTCSCK	10	HCV		LORF	1227	3.11		0.57	0.0851	
1.1062	RMVYCGVEHR	10	HCV		NS1/ENV2	632	3.11		0.27	0.012	
1.0485	HLFCHSKK	10	HCV		LORF	1390	3.11		0.27	0.025	
1.0484	TLGCGAYMSK	10	HCV		LORF	1261	3.11		0.17	0.13	
1.1067	GVCYLLPNR	10	HCV		LORF	3002	3.11		0.0029	0.032	
1.1063	LLFLADAR	10	HCV		NS1/ENV2	723	3.11		0.015	0	

Table 23(e)

Peptide	Sequence	AA	Vire	Strain	Molecule	Pos.	Motif	A1	A32	A11	A24
1.0014	FRDYVDRFY	9	HIV		CAC	298	1	0.090			
2.0129	IQYIMDOLY	9	HIV			875	1	0.064			
1.0028	TVLDVGDAY	9	HIV		POL	802	1	0.018			
1.0412	VTVLVDGDAY	10	HIV		POL	801	1	0.28	0	0.0004	
1.0415	VYQYIMDOLY	10	HIV		POL	874	1	0.25	0.0007	0.0090	
2.0252	VTVLVDGDAY	10	HIV			801	1	0.088			
1.0431	EVNIVTDGQY	10	HIV		POL	1187	1	0.053			
1.0441	LVAVHVASGY	10	HIV		POL	1329	1	0.009			
1.0442	PAETGQETAY	10	HIV		POL	1345	1	0.013			
2.0251	ISKQGFENPY	10	HIV			742	1	0.013			
2.0255	QMAVFBINPK	10	HIV			1432	3		0.61	0.64	
2.0064	RYLKDQQL	9	HIV			2778	24				0.76
2.0134	RYLKDQQL	9	HIV			2778	24				0.32
2.0065	TYQYQEPF	9	HIV			1003	24				0.30
2.0131	TYQYQEPF	9	HIV			1003	24				0.30
2.0063	TYQEPFIGNL	9	HIV			1006	24				0.052
2.0132	TYQEPFIGNL	9	HIV			1006	24				0.033
2.0064	IQYIMDOLY	9	HIV			875	24				0.013
2.0247	IYKRWILCL	10	HIV			246	24				0.017
2.0250	IYKRWILCL	10	HIV			246	24				0.014
2.0249	LYPLASLISL	10	HIV			506	24				0.014
1.0069	KLACRWPFVK	9	HIV		POL	1368	3.11		2.7	0.069	
1.0044	AVFBNFRK	9	HIV		POL	1404	3.11		0.17	1.8	
1.0082	AIHQSEMTK	9	HIV		POL	853	3.11		1.1	0.96	
1.0046	IVWQKTPK	9	HIV		POL	1075	3.11		0.085	0.37	
1.0079	KLIEDRWINK	9	HIV		VIF	1712	3.11		0.013	0.27	
1.0027	GIFPAGLJK	9	HIV		POL	788	3.11		0.23	0.065	
1.0059	QIEQLRK	9	HIV		POL	1215	3.11		0.0091	0.16	
1.0009	KIWFSYKCR	9	HIV		CAC	443	3.11		0.12	0.0005	
1.0072	ILATDQTK	9	HIV		POL	1459	3.11		0.025	0.098	
1.0036	MGYELHPDK	9	HIV		POL	925	3.11		0.064	0.086	
1.0082	YLAWVPAHK	9	HIV		POL	1227	3.11		0.077	0.067	
1.0086	KIWFSHCKR	9	HIV		CAC	443	3.11		0.077	<0.0005	
1.0047	PVNTPLVK	9	HIV		POL	1111	3.11		0.012	0.066	
1.0034	NTPVPANK	9	HIV		POL	782	3.11		0.053	0.060	
1.0080	TVQCTHCCK	9	HIV		ENV	2620	3.11		0.0021	0.046	
1.0013	ILDNQCFK	9	HIV		CAC	287	3.11		0.062	0.0068	
1.0015	RDYVDRFYK	9	HIV		CAC	299	3.11		0.0007	0.040	
1.0058	GDAQQFDK	9	HIV		POL	1199	3.11		<0.0009	0.040	
1.0064	VLRDCKK	9	HIV		POL	1254	3.11		0.088	0.032	
1.0026	LVDRELNK	9	HIV		POL	769	3.11		0.011	0.030	
1.0078	KVVPKKAKE	9	HIV		POL	1513	3.11		0.029	0.0099	
1.0042	MTKLEPFR	9	HIV		POL	839	3.11		<0.0008	0.016	
1.0463	TVYQYFVWK	10	HIV		ENV	2185	3.11		3.8	7.8	
1.0418	TVQYVLFK	10	HIV		POL	935	3.11		0.16	5.6	
1.0447	AVFBNFRK	10	HIV		POL	1404	3.11		0.66	0.85	
1.0437	KVLFLDCIK	10	HIV		POL	1253	3.11		0.36	0.78	
1.0408	KLVDFRELNK	10	HIV		POL	768	3.11		0.51	0.090	
1.0403	KLKPCMDCK	10	HIV		POL	706	3.11		0.39	0.076	
1.0395	FLGKWFYK	10	HIV		CAC	440	3.11		0.32	0.024	
1.1054	KQNFVYYR	10	HIV		POL	1474	3.11		0.082	0.21	
1.0430	GIFPAGLJK	10	HIV		POL	788	3.11		0.011	0.17	
1.0426	LVKLWYQLEK	10	HIV		POL	1117	3.11		0.054	0.082	
1.0396	MOCICCFK	10	HIV		POL	642	3.11		0.0099	0.055	
1.0413	MTKLEPFRK	10	HIV		POL	839	3.11		0.015	0.088	
1.0453	VYQONSQIK	10	HIV		POL	1504	3.11		<0.0005	0.021	
1.0394	FLGKWFYSHK	10	HIV		CAC	440	3.11		0.020	0.0013	
1.1059	IVQQONILLR	10	HIV		ENV	2741	3.11		0.0024	0.019	
1.0417	FTTPDQGHQK	10	HIV		POL	908	3.11		<0.0002	0.015	
1.0405	LVEICTEMEK	10	HIV		POL	729	3.11		0.0002	0.012	
1.0392	LVQANPDCCK	10	HIV		CAC	327	3.11		<0.0002	0.011	

Table 23(f)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A32	A11	A24
1.0225	ISEYRHICY	9	HPV	16	E6	80	1	7.8	0.0011	0.006	
1.0230	QAEPRAHY	9	HPV	16	E7	44	1	0.021			
1.0610	LQDIETCVY	10	HPV	18	E6	25	1	0.25	0.0056	0.012	
2.0159	YSKISEYRHY	10	HPV	16	E6	77	1	0.17	<0.0009	0	
2.0162	YSKISEYRHY	10	HPV	16	E6	77	1	0.11	<0.0009	0	
1.0599	HGOTPTLHEY	10	HPV	16	E7	2	1	0.007			
1.0601	QFETTDLYCY	10	HPV	16	E7	16	1	0.003			
1.0913	IHDHLECYV	10	HPV	16	E6	30	1	0.032			
2.0140	YSRRLRLHY	10	HPV	18	E6	72	1	0.018			
2.0144	YSRRLRLHY	10	HPV	18	E6	72	1	0.012			
1.0594	AVCDKCLQFY	10	HPV	16	E6	68	1	0.005			
2.0161	LURCLRCQK	10	HPV	18	E6	101	3		0.001	0.009	
2.0032	HTMLCQCK	9	HPV	18	E7	59	11		0.020	0.009	
2.0029	VYCKTVLEL	9	HPV	18	E6	33	24				0.33
2.0027	CYSLYGTIL	9	HPV	16	E6	87	24				0.057
2.0024	VYDFAPRDL	9	HPV	16	E6	49	24				0.002
2.0001	LYNLJLKL	9	HPV	18	E6	98	24				0.009
2.0000	VYGDITLEL	9	HPV	18	E6	85	24				0.010
1.0209	SVYGDITLEK	9	HPV	18	E6	84	3.11		0.39	2.3	
1.0243	SVYGDITLEK	9	HPV	18	E6	84	3.11		0.53	1.1	
1.0244	SVYGDITLEK	9	HPV	18	E6	84	3.11		0.70	0.95	
1.0226	TTLRQQYNK	9	HPV	16	E6	93	3.11		0.010	0.67	
1.0261	SRHAACHK	9	HPV	18	E6	59	3.11		0.0094	0.25	
1.0237	SRHAACHK	9	HPV	18	E6	59	3.11		0.017	0.12	
1.0233	IVCFCKQK	9	HPV	16	E7	69	3.11		0.035	0.023	
1.0997	KURHLNEKR	9	HPV	18	E6	117	3.11		0.025	<0.0005	
1.0234	LURCLRCQK	9	HPV	18	E6	102	3.11		0.019	0.0012	
1.0653	ILECVYCK	9	HPV	16	E6	33	3.11		0.0016	0.009	
1.0999	CIDFYSRL	9	HPV	18	E6	48	3.11		0.017	0.0016	
1.0998	CIDFYSRL	9	HPV	18	E6	48	3.11		0.010	0.0009	
1.0996	CTTLRQQYNK	10	HPV	16	E6	92	3.11		0.010	0.98	
1.0606	LURCLRCQK	10	HPV	18	E6	101	3.11		0.076	0.29	
1.0998	LURCLRCQK	10	HPV	16	E6	106	3.11		0.12	0.34	
1.0629	LURCLRCQK	10	HPV	18	E6	101	3.11		0.16	0.11	
1.0614	LTEVFEPAK	10	HPV	18	E6	41	3.11		0.0009	0.11	
1.0605	GVCPICQK	10	HPV	16	E7	88	3.11		0.0017	0.060	
1.0625	LTEVFEPAK	10	HPV	18	E6	41	3.11		0.0012	0.041	
1.0591	DMLECVYCK	10	HPV	16	E6	32	3.11		0.0065	0.001	
1.1101	KURHLNEKR	10	HPV	18	E6	117	3.11		0.013	0	
1.1095	CVYCKQLLR	10	HPV	16	E6	37	3.11		0.011	0.0059	

Table 23(9)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
2.0020	EVDPICHLY	9	MAGE	3		161	1	18	0.0002	0.0009	
3.0172	EADPTISNTY	9	MAGE	5/51		161	1	9.9	0.0006	0.0026	0
1.0258	LTQDLVQEKY	9	MAGE	1		240	1	2.1	0	0.0002	
3.0173	EVDPICHVY	9	MAGE	6		161	1	1.9	<0.0002	<0.0002	0
1.0254	EADPTCHSY	9	MAGE	1		161	1	1.1	0	0	
1.0259	LVQEKYLEY	9	MAGE	1		243	1	0.42	0.0013	0.053	
6.0063	TSYVKVLEY	9	MAGE	1	new	275	1	0.099			
2.0009	SSLPTTINY	9	MAGE	3		9	1	0.055			
2.0011	GSVVGNWQY	9	MAGE	3		77	1	0.060			
2.0008	SSPTTINY	9	MAGE	2		9	1	0.043			
1.0252	MLESVINY	9	MAGE	1		128	1	0.011			
2.0147	ASLPTTINY	10	MAGE	3		8	1	2.6	<0.0009	0.033	
2.0147	LTQDLVQEKY	10	MAGE	1		239	1	1.2	<0.0009	0.0073	
6.0114	ETSYVKVLEY	10	MAGE	1	new	274	1	0.56			
2.0141	ASSPTTINY	10	MAGE	2		8	1	0.17	<0.0009	0.026	
1.0648	DLVQEKYLEY	10	MAGE	1		242	1	0.044			
6.0045	TSYVKVLEY	9	MAGE	1	new	275	3		0.71	0.010	
4.0119	TTNFTQK	9	MAGE	1		66	3		0.043	0.37	
6.0064	ALAETSYVK	9	MAGE	1	new	271	3		0.31	0.36	
6.0132	LTQDLVQEK	9	MAGE	1		239	3		<0.0009	0.14	
6.0062	LVQEKYLEY	9	MAGE	1	new	243	3		0.0026	0.034	
4.0131	HSAYCEPIK	9	MAGE	1		229	3		0.084	0.0009	
4.0122	LFRAVITK	9	MAGE	1		97	3		0.011	0.0005	
6.0124	RVRFPFSLR	10	MAGE	1	new	230	3		0.43	0.0099	
6.0161	ADLVCFLLK	10	MAGE	1		107	3		0.35	0.29	
6.0160	ESLFAVITK	10	MAGE	1		95	3		0.14	0.088	
6.0119	DLVQEKYLEY	10	MAGE	1	new	242	3		0.032	0.0051	
6.0123	YVVKVSARVR	10	MAGE	1	new	283	3		0.019	0.0009	
4.0148	LSVMEVYDGR	10	MAGE	1		218	3		<0.0009	0.012	
4.0143	KASMLESVIK	10	MAGE	1		125	3		<0.0003	0.0097	
6.0125	RALAETSYVK	10	MAGE	1	new	270	11		0.18	0.24	
2.0010	NYPLWSQSY	9	MAGE	3		16	24				0.027
2.0145	NYKHCFEIP	10	MAGE	1		135	24				0.25
2.0151	LYPATCLCL	10	MAGE	3		115	24				0.048
6.0126	SYVKVLEYVI	10	MAGE	1	new	276	24				0.006
1.0248	SLFAVITK	9	MAGE	1		96	3.11		4.1	2.7	
1.1006	SVMEVYDGR	9	MAGE	1		219	3.11		0.0083	1.3	
1.1004	TTNFTQK	9	MAGE	1		66	3.11		0.016	1.0	
1.0257	LTQDLVQEK	9	MAGE	1		239	3.11		0.0002	0.38	
1.0634	SLFAVITK	10	MAGE	1		96	3.11		1.2	0.98	
1.0647	LLTQDLVQEK	10	MAGE	1		238	3.11		0.0004	0.16	
1.0640	MLESVINYK	10	MAGE	1		128	3.11		0.14	0.027	
1.0644	LLGDNQMPK	10	MAGE	1/3		182	3.11		0.020	0.011	
1.0630	SLEORSUHCX	10	MAGE	1		2	3.11		0.015	0.015	

Table 23(h)

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0281	GSDCTTHY	9	p53			226	1	29.5	0.0010	0.029	
1.0467	GTAHSVICTY	10	p53			117	1	0.33	0.023	0.049	0
1.0572	RVEGNLRVEY	10	p53			196	1	0.022			
1.0278	RVRAMATYK	9	p53			156	3.11		1.5	0.73	
1.0276	CTYSPALNK	9	p53			124	3.11		0.44	1.1	
1.0285	NTSSSPOPK	9	p53			311	3.11		0.0009	0.095	
1.0284	RTEENLRK	9	p53			283	3.11		0.0015	0.091	
1.0287	ELNEALEK	9	p53			343	3.11		0.020	0.0062	
1.0678	RTEENLRKK	10	p53			283	3.11		3.3	0.0080	
1.1113	KTYQGSYCFR	10	p53			101	3.11		2.6	0.88	
1.1115	VVRRCPTHER	10	p53			172	3.11		0.099	0.0017	
1.0679	NTSSSPOPKK	10	p53			311	3.11		0.0035	0.064	
1.1121	KVCAQCFDR	10	p53			273	3.11		0.014	0.011	
1.1116	GLAPFOELR	10	p53			187	3.11		0.013	0.0006	

Table 23(i)

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Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Metif	A1	A3.2	A11	A24
3.0175	KCEYFVEMY	9	PAP			322	1	3.4	<0.0002	0.0002	0
3.0174	LCEYBKKY	9	PAP			81	1	0.78	<0.0002	0.0002	0
3.0166	ASCHLTLY	9	PAP			311	1	0.77	<0.0002	0.055	0
3.0163	ESYKHEQVY	9	PAP			95	1	0.098	<0.0002	0.0002	0
3.0237	LSLSRLSLY	10	PAP			238	1	14	0.0026	0.0004	0
3.0235	LSLSRLSLY	10	PAP			238	1	12	0.0005	0.0004	0
3.0236	LTQLCMHQHY	10	PAP			70	1	0.62	0.015	0.0024	0.0022
3.0238	KCEYFVEMY	10	PAP			322	1	0.018			
3.0230	LVNELNHHMK	10	PAP			263	3		0.056	0.12	
3.0158	ATQPSYCK	9	PAP			274	11		0.10	1.2	
3.0231	ETLKSEEROK	10	PAP			170	11		<0.0004	0.014	
3.0161	LYPERCEYF	9	PAP			318	24				2.5
3.0160	LYCESVHNF	9	PAP			213	24				0.44
3.0159	PYKDRATL	9	PAP			183	24				0.11
3.0162	VYNGLLFPY	9	PAP			302	24				0.032
3.0232	PYASCHLTSL	10	PAP			309	24				0.024

Table 23(j)

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Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	Motif	A1	A3.2	A11	A24
1.0220	ALPERPSLY	9	PSA			231	1	0.011			
2.0137	VSEPPHPLY	10	PSA			88	1	0.15	<0.0003	0.0015	
1.0265	PLYDMSLLK	9	PSA			95	3.11		0.24	0.037	
1.0273	VVHYKWK	9	PSA			242	3.11		0.0072	0.003	
1.0272	YTKVVHYRK	9	PSA			239	3.11		0.0006	0.004	
1.1009	SLIGNRRLR	9	PSA			100	3.11		0.0024	0.047	
1.0260	IVCGWCEK	9	PSA			21	3.11		0.041	0.019	
1.0269	QVHPQKVTK	9	PSA			182	3.11		0.0060	0.014	
1.1112	SLYTKVVHYR	10	PSA			227	3.11		0.28	0.23	
1.0463	LTAAHQDNK	10	PSA			57	3.11		0.14	0.003	
1.0461	RVCGWCEK	10	PSA			20	3.11		0.046	0.067	
1.0462	KVVHYKWK	10	PSA			241	3.11		0.045	0.045	
1.1111	VTKPMLCGR	10	PSA			189	3.11		0.0003	0.012	
3.0108	MLRLSEPA	9	PSA			118	Random				

Table 23(k)

SENT BY:

8- 6-93 : 15:27 :

CYTEL-

415 543 5043:#25/25

TABLE 24: CTL EPTOPES IDENTIFIED IN PEPTIDE SCREENING.

Sequence	Antigen	Motif	Id
EVDPIGHLY	MAGE3	A01	1044.07
ASSLEPTNMY	MAGE3	A01	1044.01
EADPTGHSY	MAGE1	A01	958.01
SSLEPTNMY*	MAGE3	A01	1072.02*
GSVVGNMAY*	MAGE3	A01	1072.03*
ALAETSIVK*	MAGEIN	A03	1072.38*
SLFRAVITK	MAGE1	A11	1072.13
RALAETSIVK	MAGEIN	A11	1072.39
ESLFRVITK	MAGE1	A11	1072.15
KVYLAMPVPAHK	HIV	A3/11*	1069.42*
TVYGVVPVK	HIV	A03	1069.43
KLGRMPVK	HIV	A03	1069.44
KMIGIGGFIK	HIV	A03	1069.45
AIFQSSMTK	HIV	A03	966.01
WTYQIYQEPFK	HIV	A03	1069.46
ELGKIWPSHK*	HIV	A03	1069.56*
TVYGVVPVK	HIV	A11	1052.03
VTVYGVVPVK	HIV	A11	1069.47
GVAGALVAFK	HCV	A03	1073.10
CTCGSSDLY	HCV	A11	1069.62
GVAGALVAFK	HCV	A11	1052.05
LIDTASALY*	HBV	A01	1069.01*
TLWKAGILYK	HBV	A03	1069.15
* borderline positive			

Table 25 a
Peptides Synthesized
by Cytel For Loading
Onto Acid Stripped
Autologous PBMCs and
PHA Blasts

	Peptide ID #	Antigen	Sequence
	777.03	HBs	FLLTRILTI
10	924.07	HBc	FLPSDFFPSV
	927.32	HBp	GLYSSTVPV
	938.01	MAGE 1	EADPTGHSY
	939.03	PSA	VLVHPQWVL
	941.01	HBc	FLPSDYFPSV
15	1044.04	PAP	ILLWDPIPV
	1044.05	PSA	KLQCVDLVHI
	1044.06	PSA	MLLRLSEPAEL

20 Table 25 b
Cell Population

	¹²⁵ I-Labeled	CPMS	
	Peptide +/- Cold	+/- std. dev.	
	Peptide		
	- cold peptide	3553 ± 157	n = 3
25	+ cold peptide	13	n = 1
	-cold peptide	370 ± 37	n = 3
	+ cold peptide	50	n = 1

WHAT IS CLAIMED IS:

1. A composition comprising an immunogenic peptide having a HLA-A3.2 binding motif, which immunogenic peptide has
5 between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue selected from the group consisting of L, M, I, V, S, A, T, F, C, G, D and E;

10 and a second conserved residue of K, R, Y, H and F;
wherein the first and second conserved residues are separated by 6 to 7 residues.

2. The composition of claim 1, wherein the first conserved residue is at the second position from the
15 N-terminus.

3. A composition comprising an immunogenic peptide having a HLA-A1 binding motif, which immunogenic peptide has
20 between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue of T, S and M; and

a second conserved residue of D, E, A, S and T;

a third conserved residue of Y;

25 wherein the first and second conserved residues are adjacent and the second and third conserved residues are separated by 5 or 6 residues.

4. The composition of claim 3, wherein the first conserved residue is at the second position from the
30 N-terminus.

5. A composition comprising an immunogenic peptide having a HLA-A1 binding motif, which immunogenic peptide has
35 between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue of T, S and M; and

a second conserved residue of Y;

wherein the first and second conserved residues are separated by 6 to 7 residues.

5 6. The composition of claim 5, wherein the first conserved residue is at the second position from the N-terminus and the second conserved residue is at the ninth or tenth position from the N-terminus.

10 7. A composition comprising an immunogenic peptide having an HLA-A1 binding motif, which immunogenic peptide has between 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus.

 a first conserved residue of D, E, A, S and T; and
 a second conserved residue of Y;
15 wherein the first and second conserved residues are separated by 5 to 6 residues.

20 8. The composition of claim 5, wherein the first conserved residue is at the third position from the N-terminus and the second conserved residue is at the ninth or tenth position from the N-terminus.

25 9. A composition comprising an immunogenic peptide having a HLA-A11 binding motif, which peptide has between about 9 and about 10 residues and the following residues, from the Nterminus to the C-terminus:

 a first conserved residue of L, M, I, V, A, S, T, G,
 N, Q, C, F, D, E; and
 a second conserved residue of K, R, H;
30 wherein the first and second conserved residues are separated by 6 to 7 residues.

35 10. The composition of claim 9, wherein the first conserved residue is at the second position from the N-terminus.

 11. A composition comprising an immunogenic peptide having a HLA-A24.1 binding motif, which immunogenic peptide

has between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue of Y, F, W; and

a second conserved residue of F, I, L, W, M;

5 wherein the first and second conserved residues are separated by 6 to 7 residues.

12. The composition of claim 11, wherein the first conserved residue is at the second position from the
10 N-terminus.

13. A composition comprising an immunogenic peptide having an HLA-A3.2 binding motif, which immunogenic peptide has 9 or 10 residues:

15 a first conserved residue at the second position selected from the group consisting of A, I, L, M, T, and V; and a second conserved residue at the C terminal position selected from the group consisting of K and R.

 wherein the first and second conserved residues are
20 separated by 6 to 7 residues.

14. A composition comprising an immunogenic peptide having an HLA-A11 binding motif, which immunogenic peptide has 9 or 10 residues and the following residues, from the
25 N-terminus to the Cterminus:

a first conserved residue at the second position from the N terminus selected from the group consisting of A, I, L, M, T and V; and

30 a second conserved residue at the C terminal position selected from the group consisting of K;

 wherein the first and second conserved residues are separated by 6 to 7 residues.

15. A pharmaceutical composition comprising a
35 pharmaceutically acceptable carrier and an immunogenic peptide having a HLA-A3.2 binding motif, which immunogenic peptide has between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue selected from the group consisting of L, M, I, V, S, A, T, F, C, G, D and E; and a second conserved residue of K, R and Y; wherein the first and second conserved residues are separated by 6 to 7 residues.

16. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an immunogenic peptide having a HLA-A1 binding motif, which immunogenic peptide has between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue of T, S and M; and
a second conserved residue of D, E, A, S and T;
a third conserved residue of Y;

wherein the first and second conserved residues are separated by 1 residue and the second and third conserved residues are separated by 5 or 6 residues.

17. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an immunogenic peptide having a HLA-A1 binding motif, which immunogenic peptide has between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue of T, S or M; and
a second conserved residue of Tyr;

wherein the first and second conserved residues are separated by 6 to 7 residues.

18. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an immunogenic peptide having a HLA-A1 binding motif, which peptide has between about 9 and about 10 residues and the following residues, from the N-terminus to the C-terminus:

a first conserved residue of D, E, S, T; and
a second conserved residue of Y;

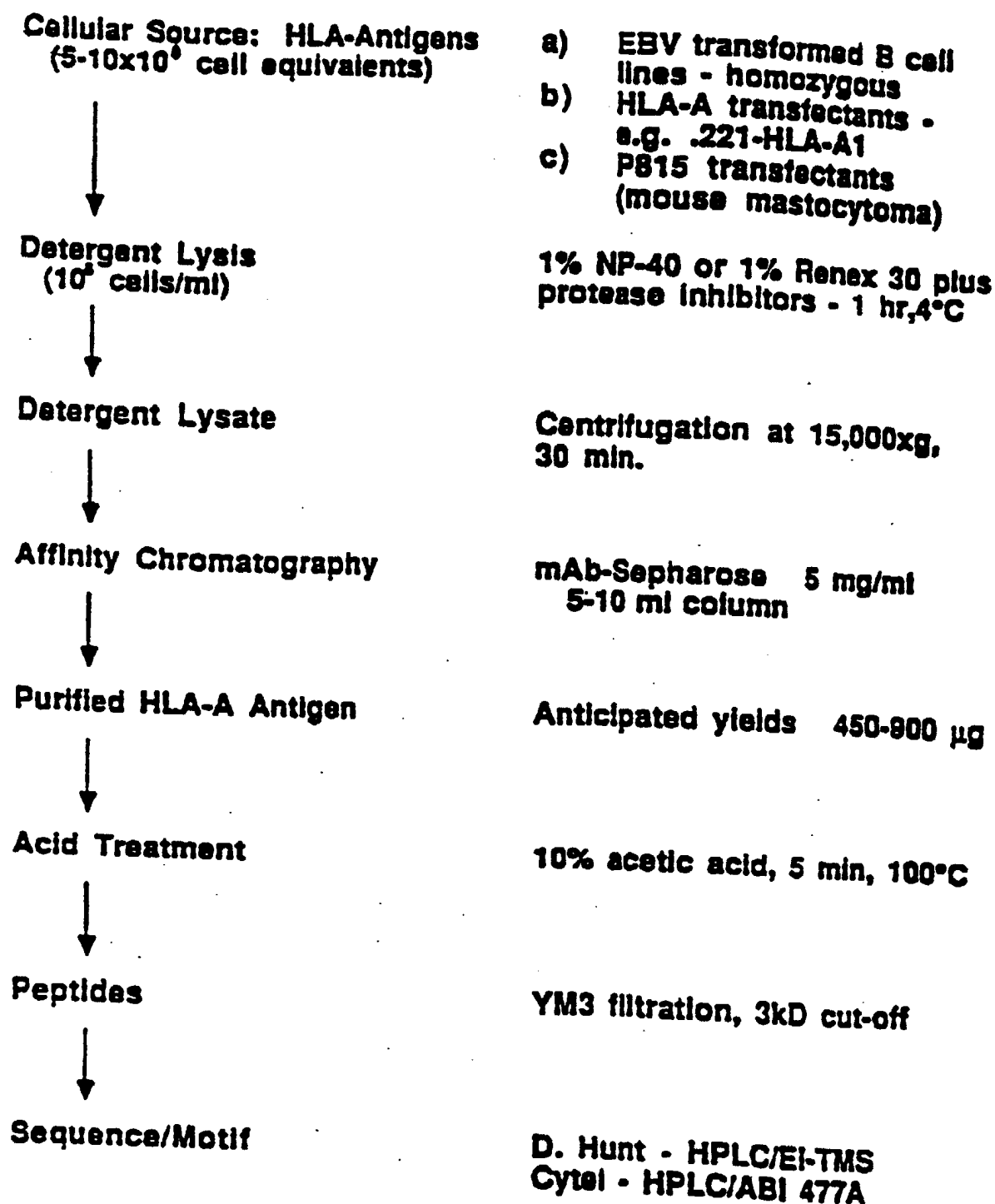
wherein the first and second conserved residues are separated by 5 to 6 residues.

19. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an immunogenic peptide having a HLA-A24.1 binding motif, which peptide has
a first conserved residue of Y, F, W; and
5 a second conserved residue of F, I, L, W, or m;
wherein the first and second conserved residues are separated by 6 to 7 residues.

20. A method of identifying an immunogenic peptide
10 comprising the following steps:
determining a binding motif for an MHC molecule encoded by a preselected MHC Class I allele;
screening an amino acid sequence of an antigenic protein for the presence of the binding motif;
15 selecting a sequence in the antigenic protein having the binding motif;
preparing a test peptide of about 8 and about 11 residues comprising the selected subsequences;
determining the ability of the test peptide to bind
20 to the preselected MHC allele and induce a CTL response, thereby identifying immunogenic peptides.

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HLA-A PURIFICATION AND PEPTIDE ELUTION



4/6/92:RK

Figure 1

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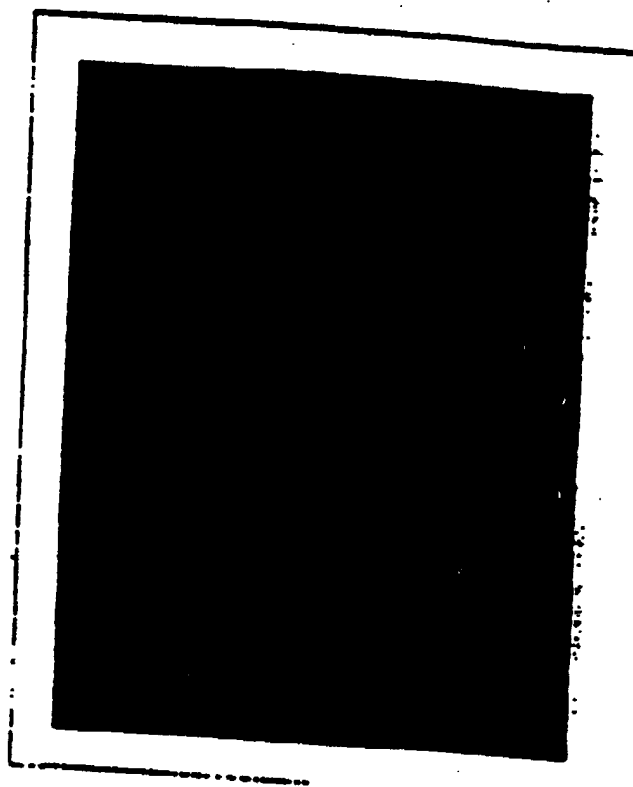


Figure 2

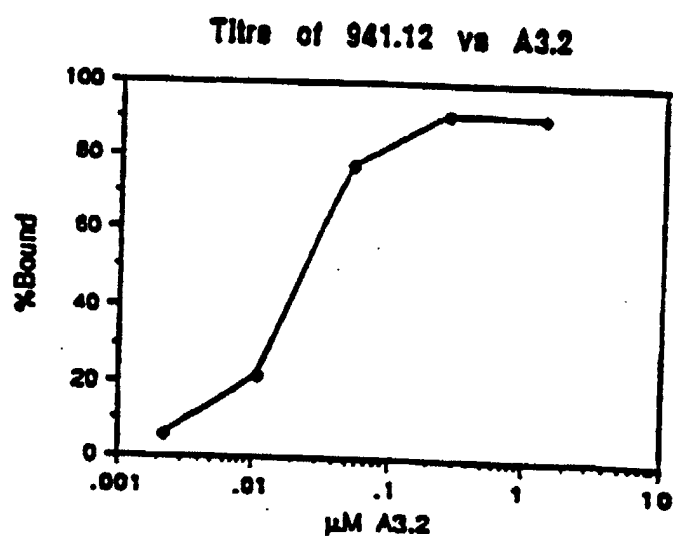


Figure 4

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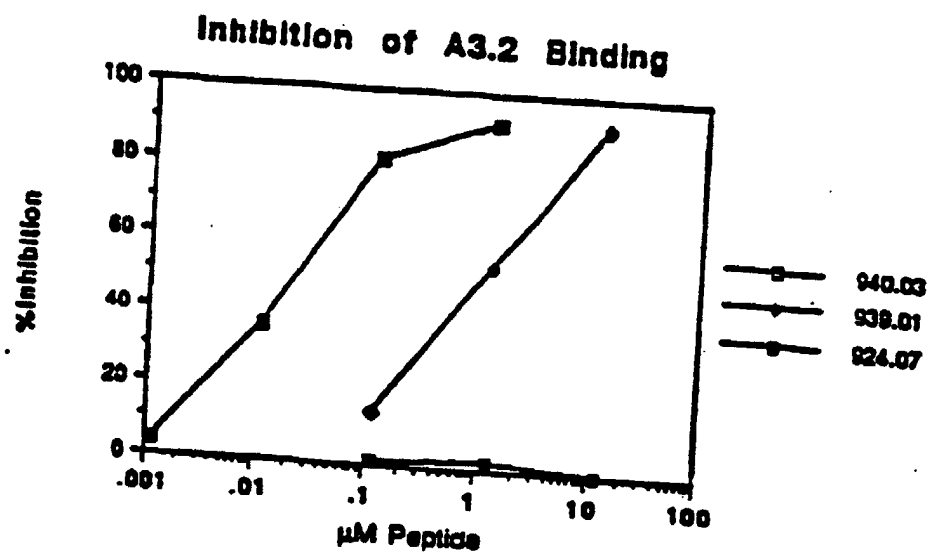


Figure 5

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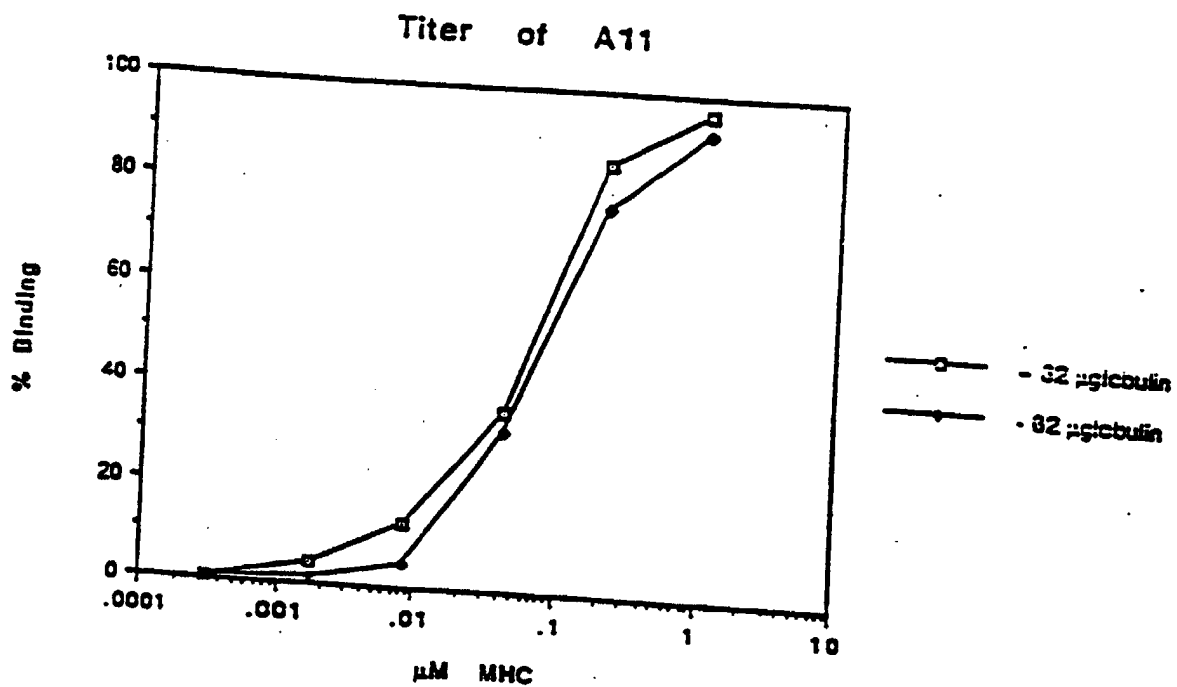


Figure 6

7/25

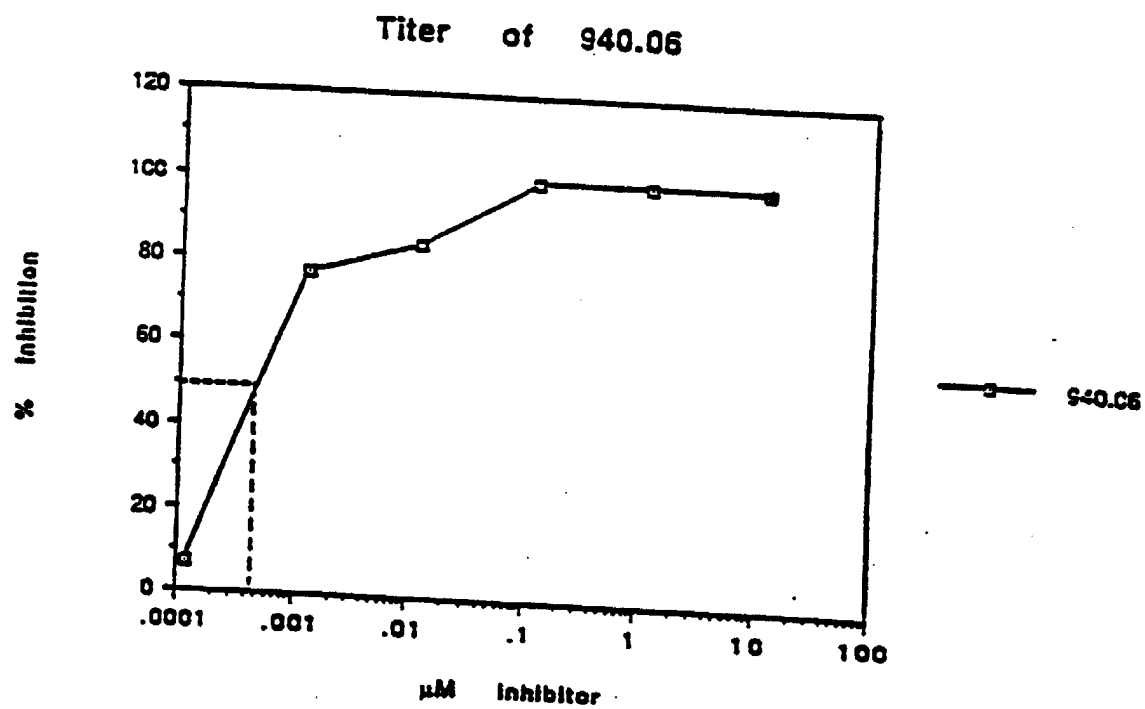


Figure 7

8/25

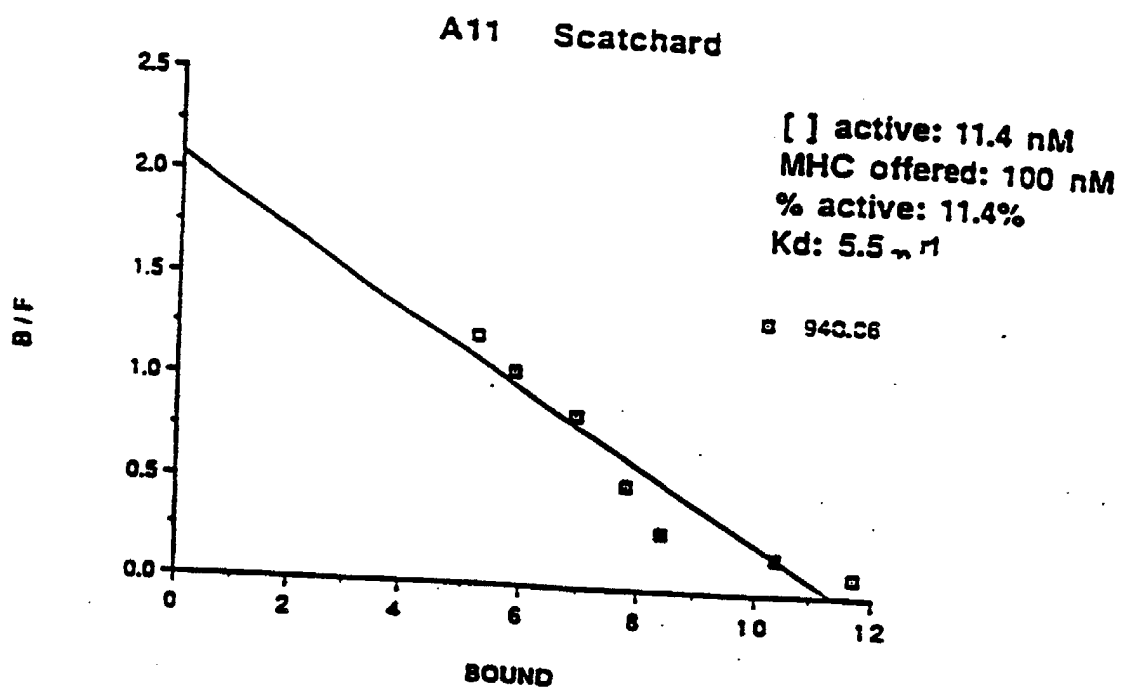


Figure 8

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Titer of 944.02 against A1.

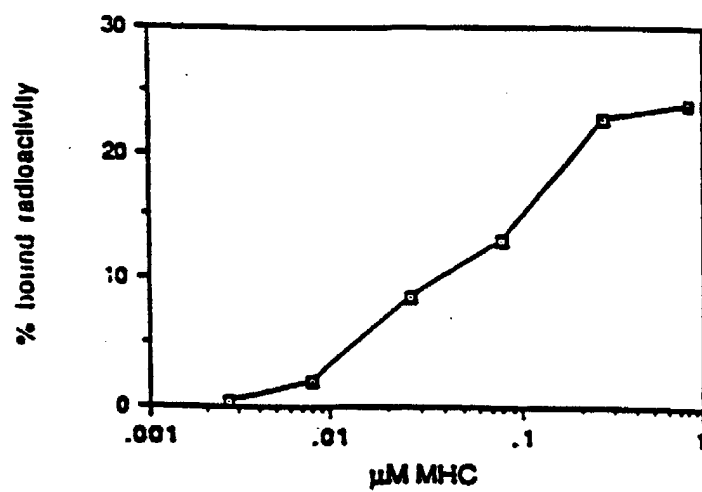


Figure 9

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AI inhibition titration.

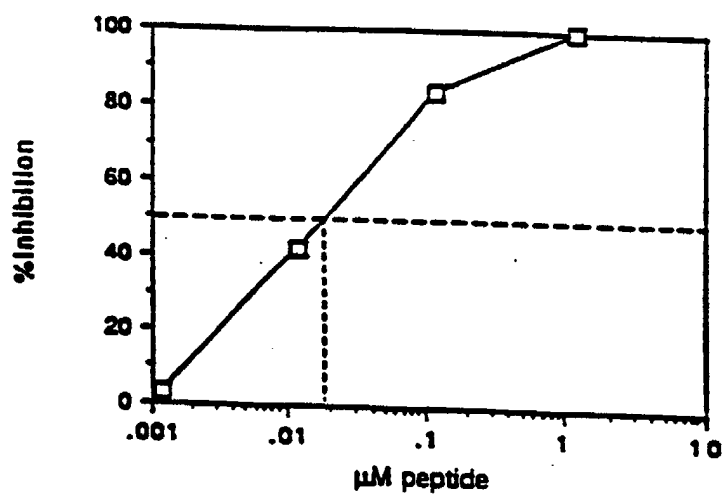


Figure 10

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Scatchard for A1 vs. 944.02

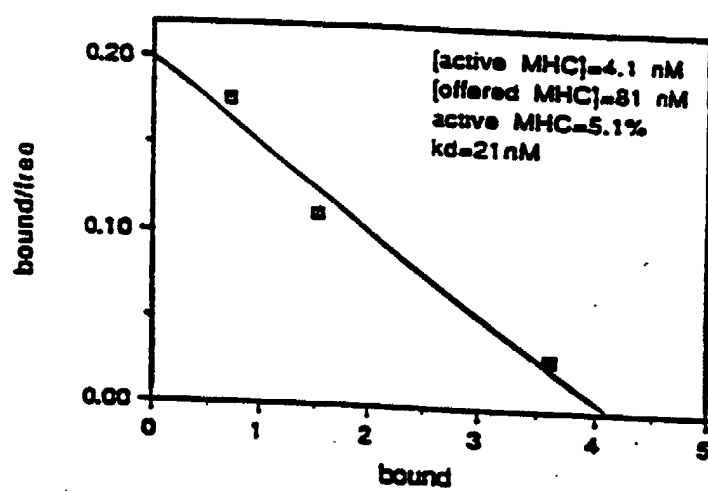


Figure 11

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A24 titration.

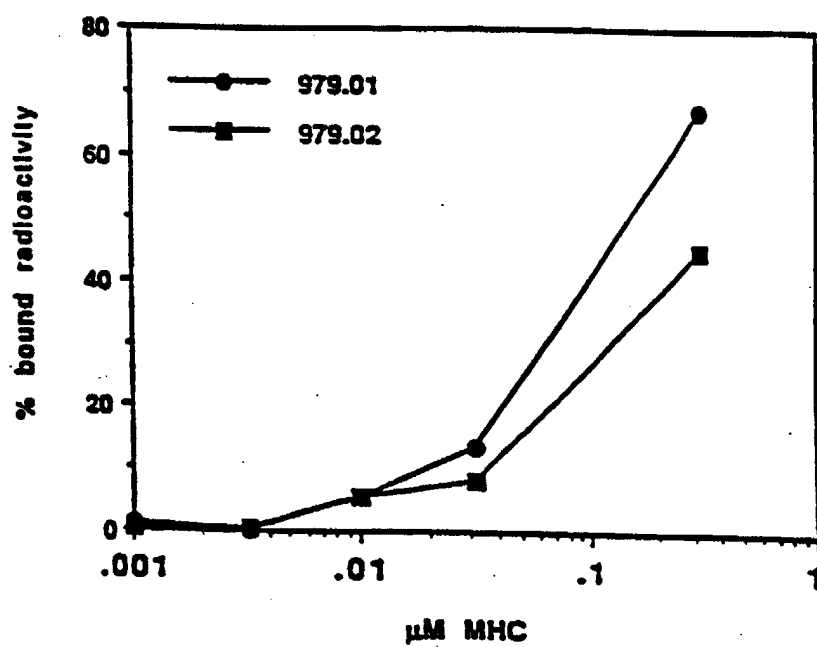


Figure 12

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A24 Inhibition.

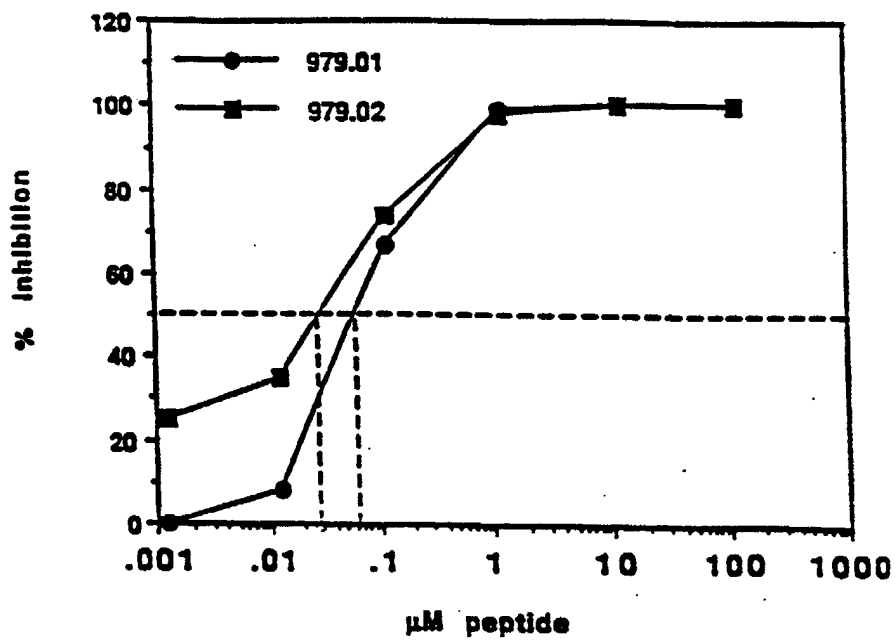


Figure 13

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Figure 14A

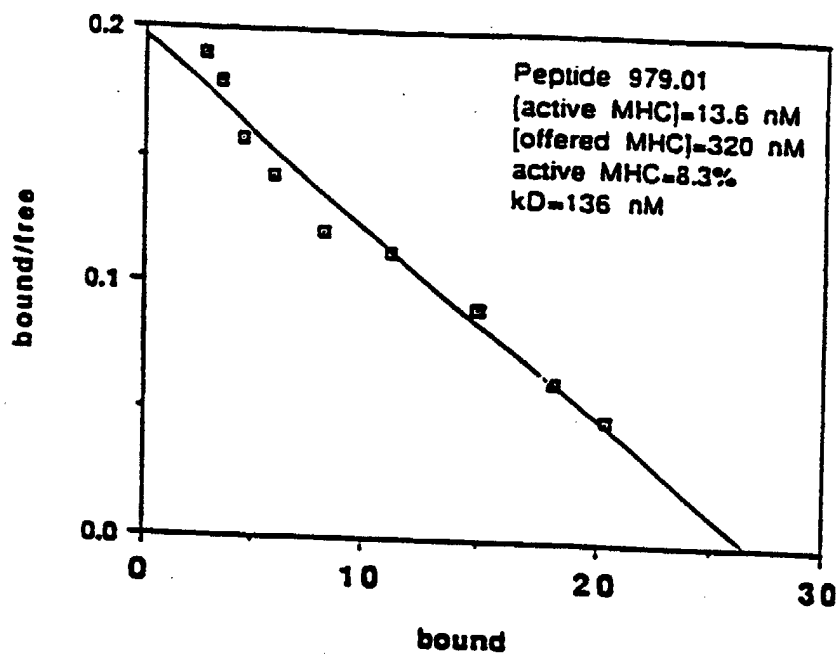


Figure 14B

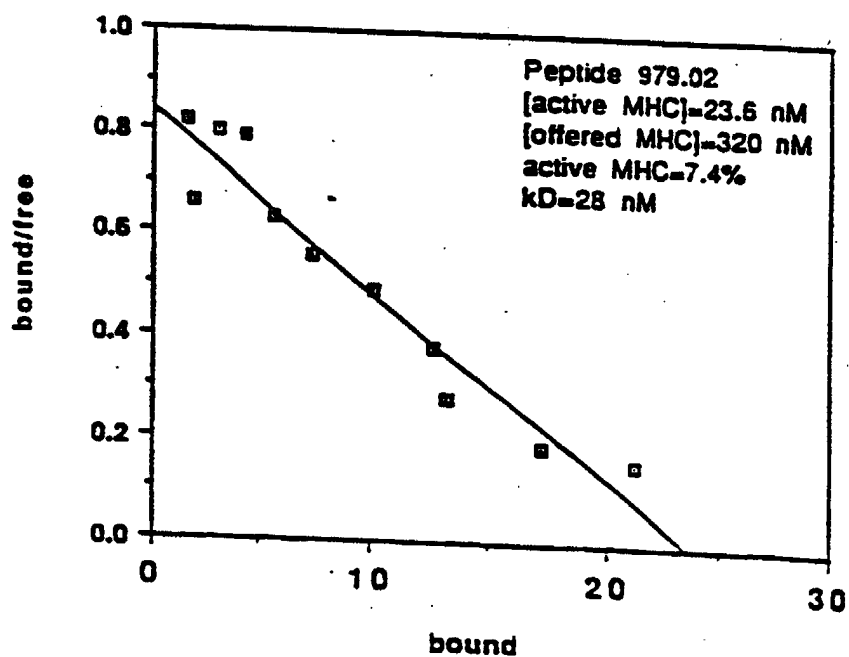
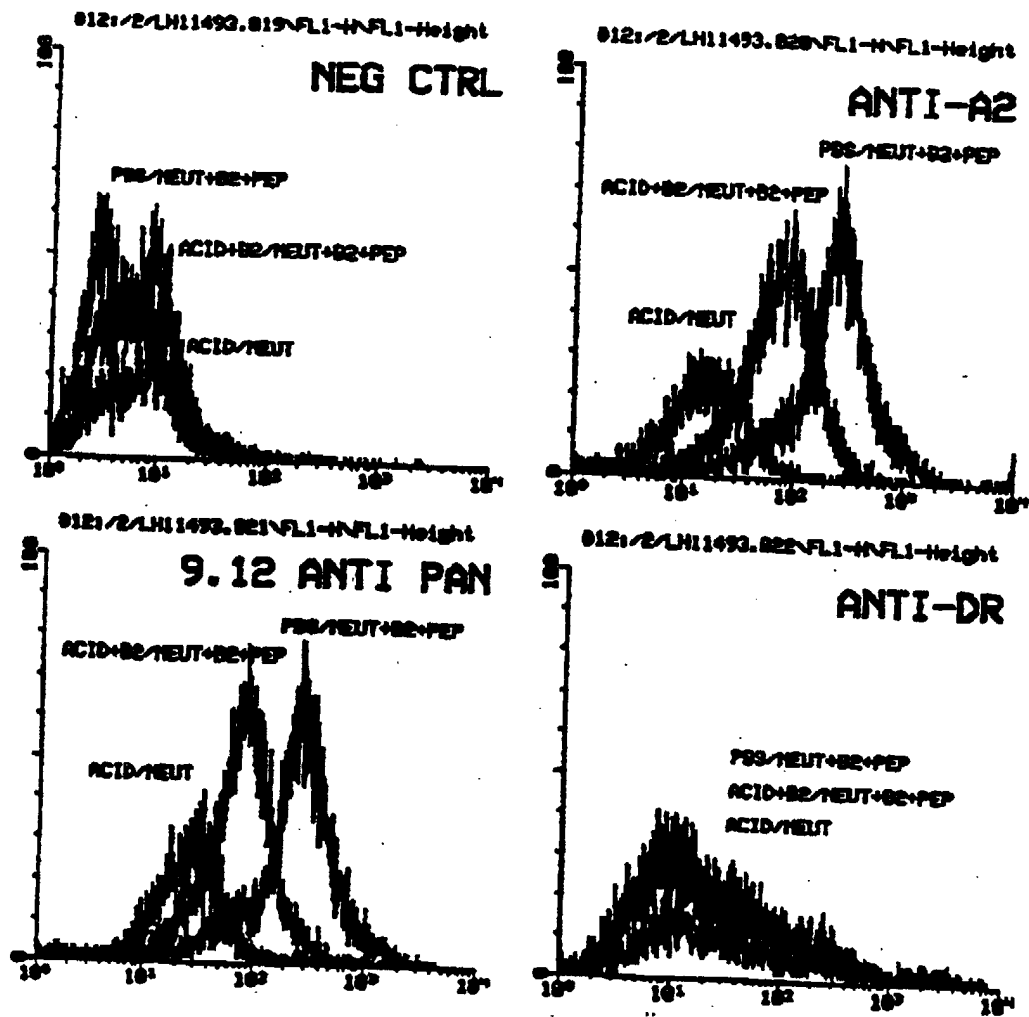


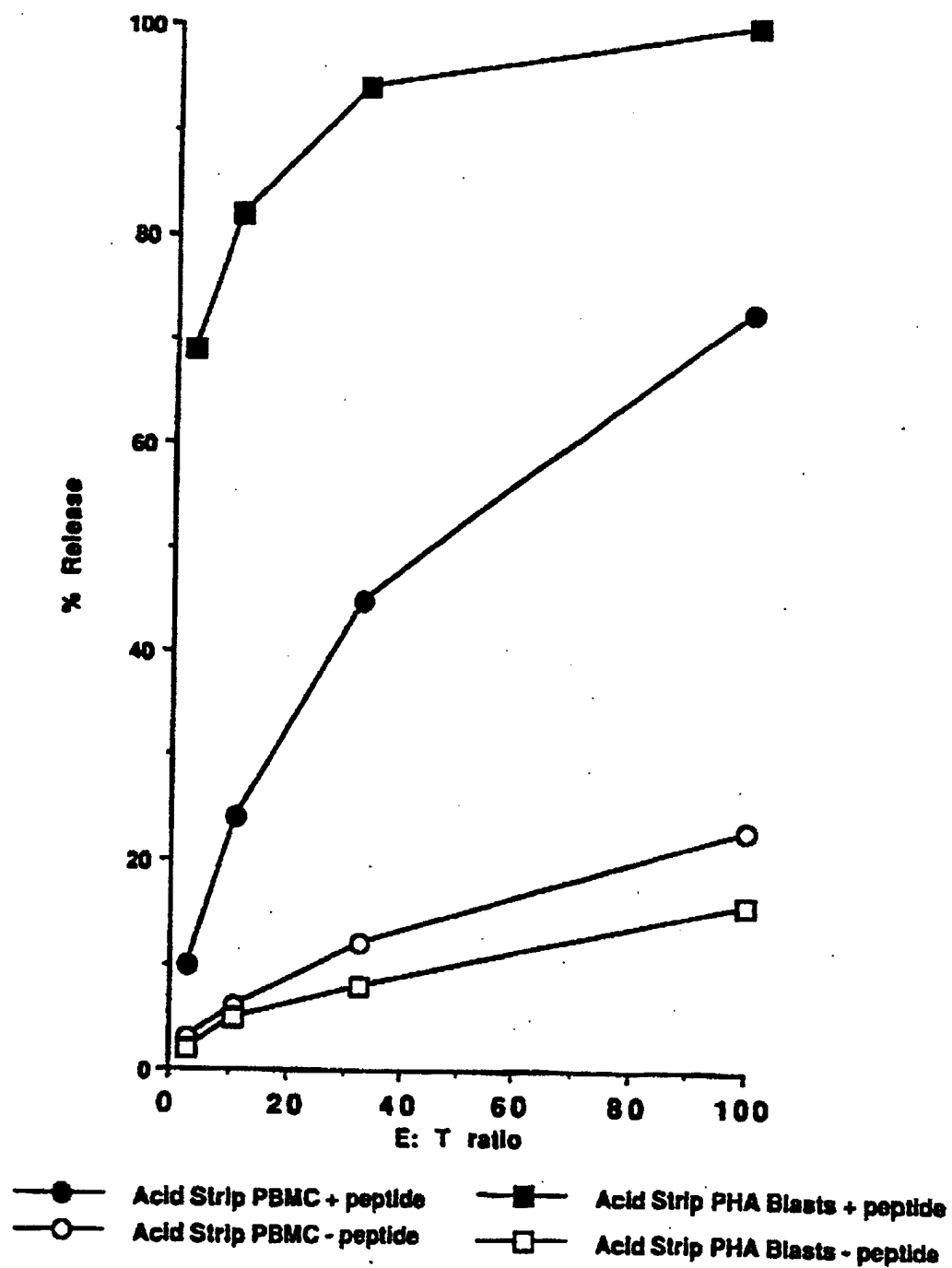
Figure 15



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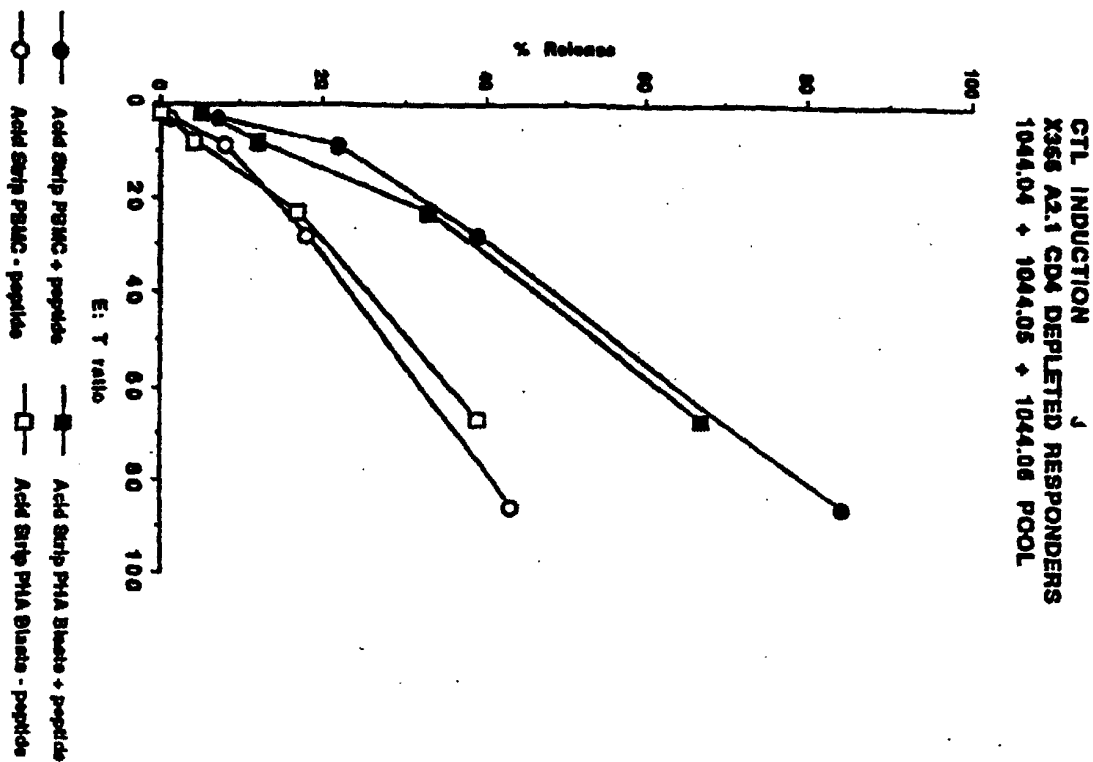
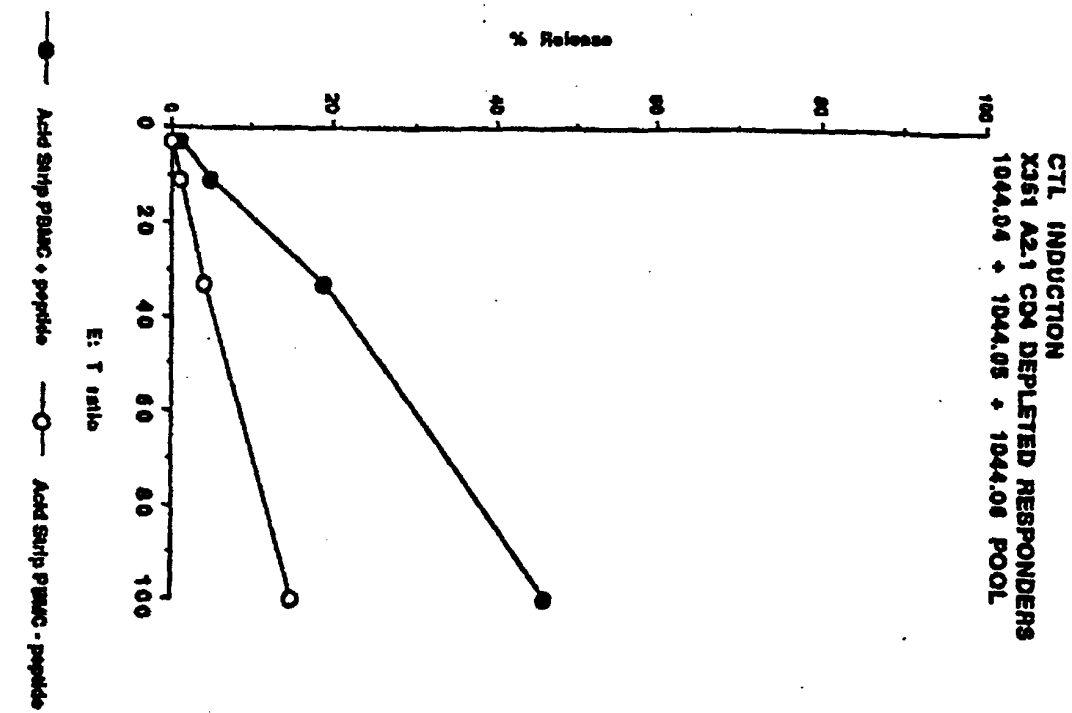
Figure 16

CTL INDUCTION
GC43 A2.1 CD4 DEPLETED RESPONDER
777.03 + 924.07 + 927.32 POOL



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Figure 17



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Figure 18

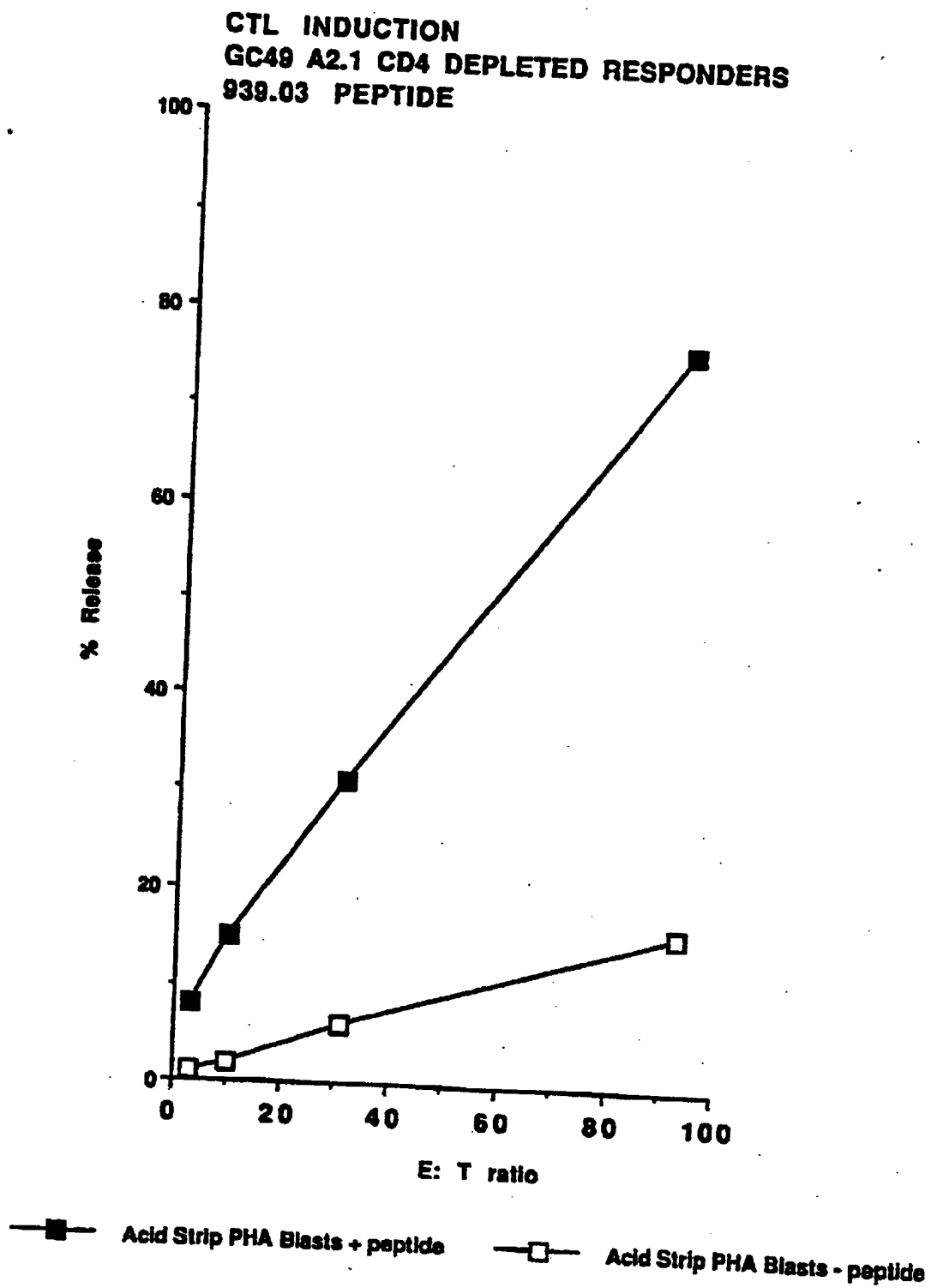
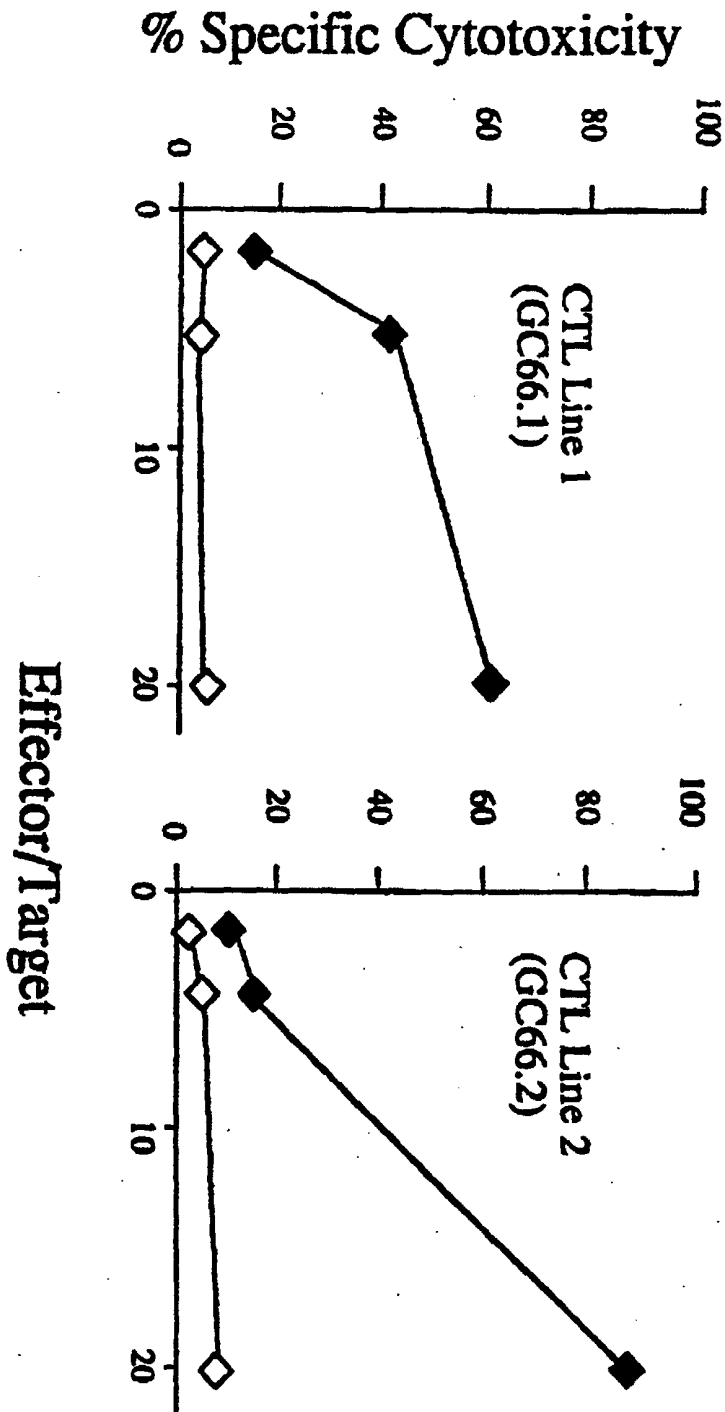


Figure 19

MAGE1 Peptide Specific, HLA-A1-Restricted CTL Can Kill Melanoma Cells Expressing the Endogenous Antigen



- ◆ 938-mel (HLA-A1/24, MAGE1⁺)
- ◇ 888-mel (HLA-A1/24, MAGE1⁻)

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Figure 20

**Cytotoxic Activity of an HLA-A1-Restricted CTL Line
Specific for a MAGE3 Peptide (1044.07)**

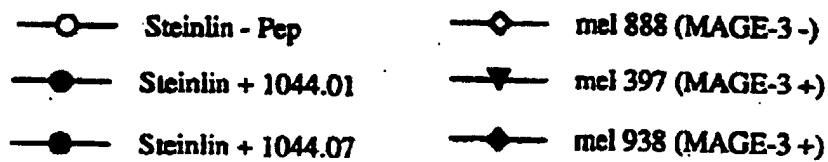
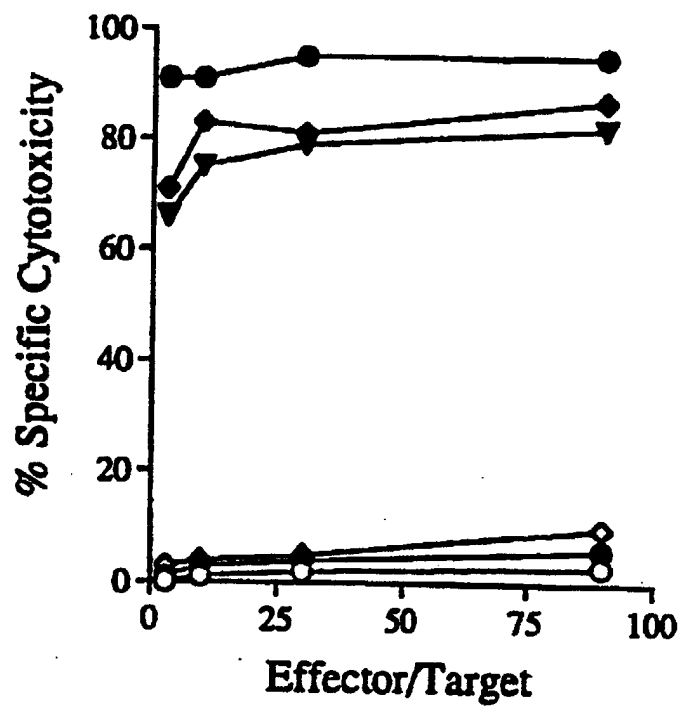
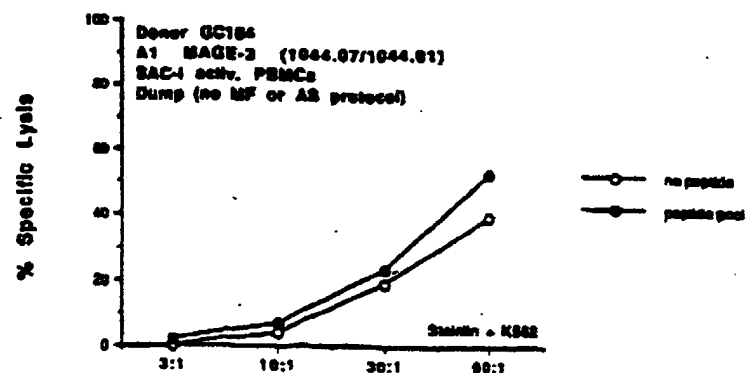
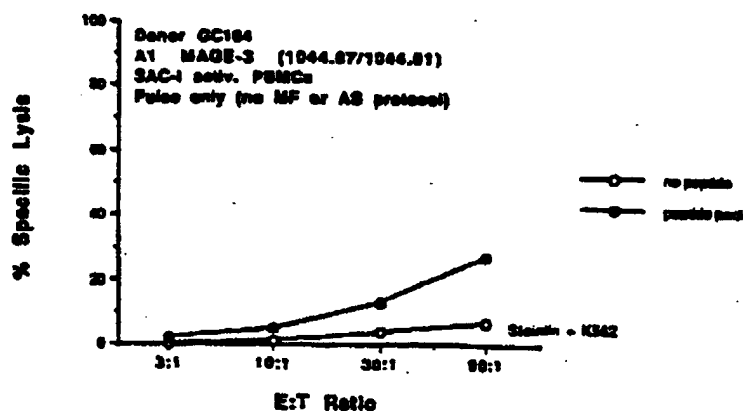
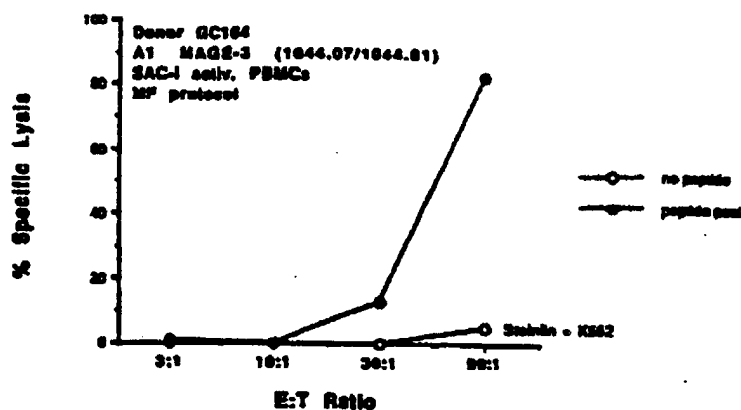
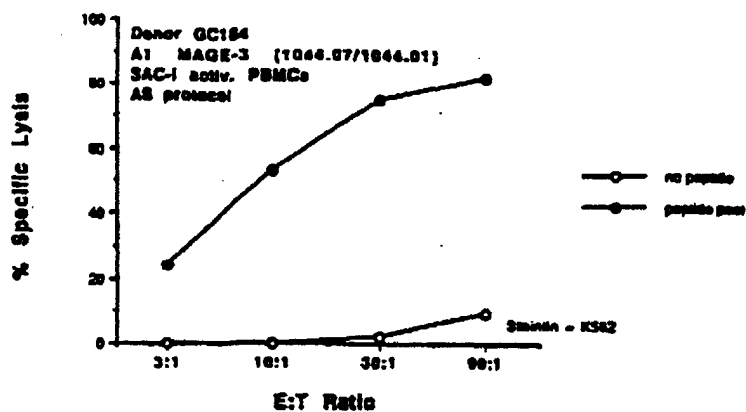


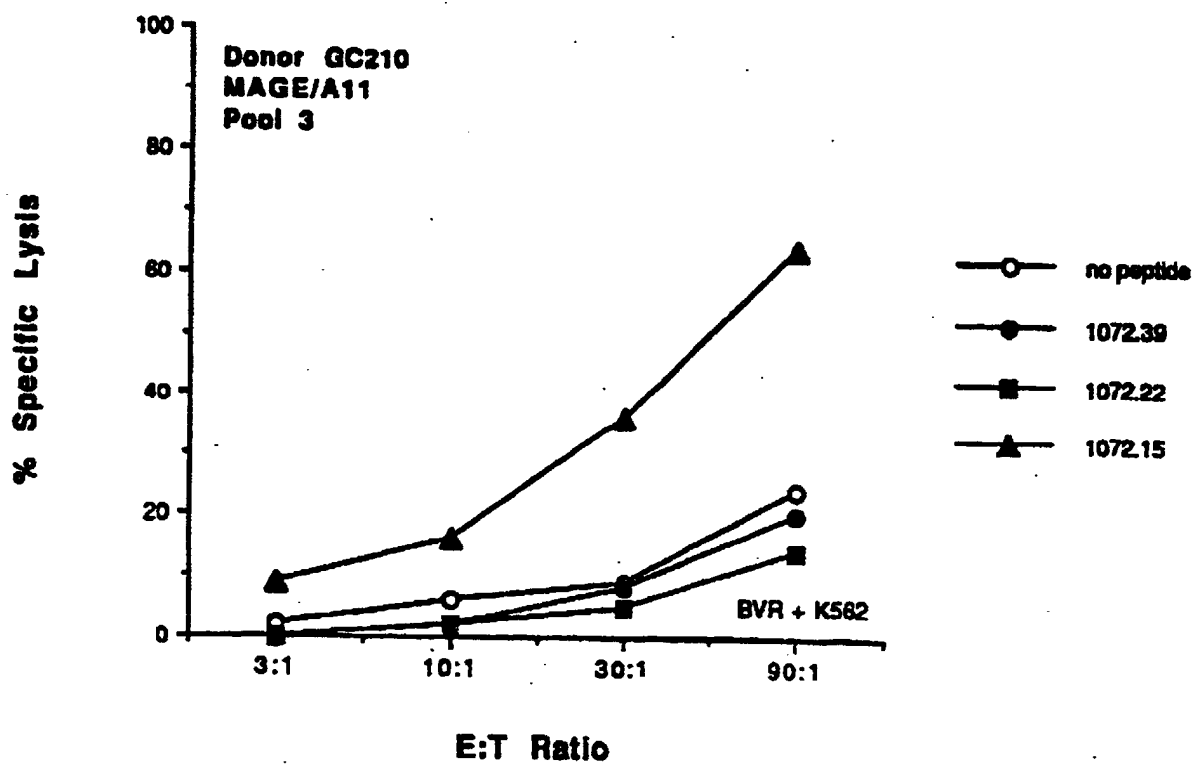
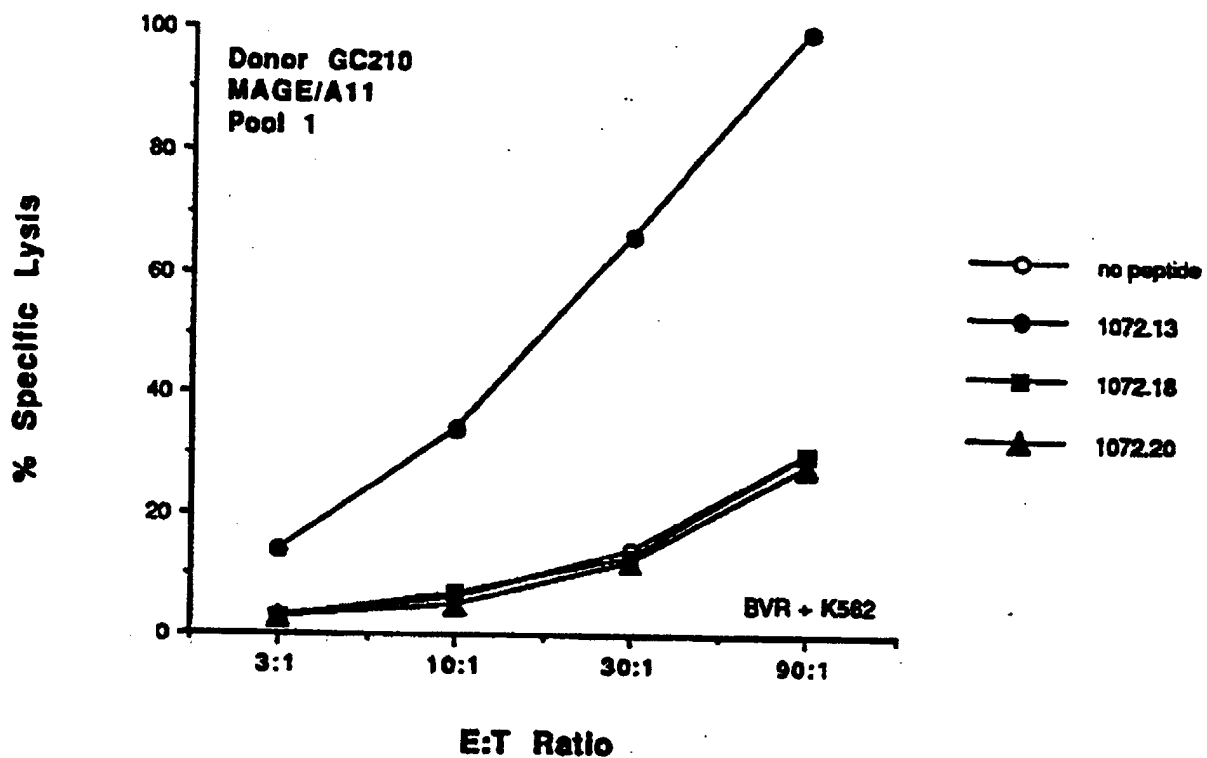
Fig. 21

Induction Using Different Methods to Load Peptides onto SAC-I Cells.



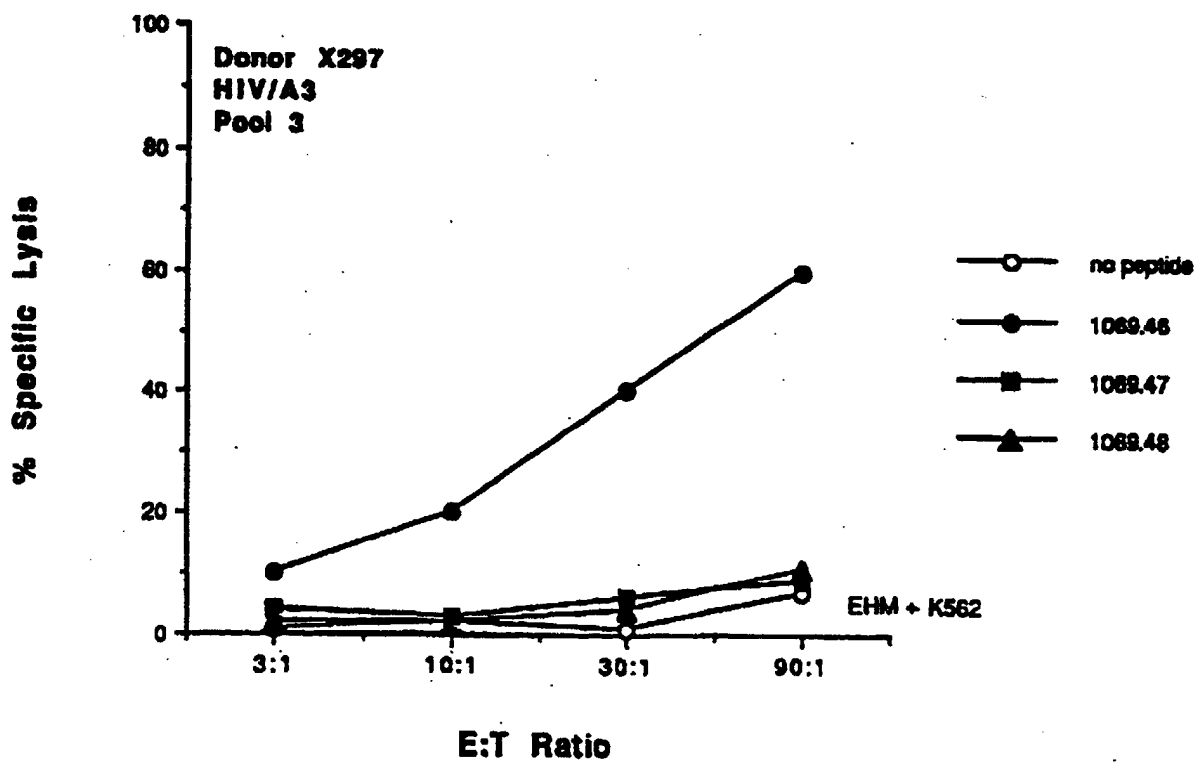
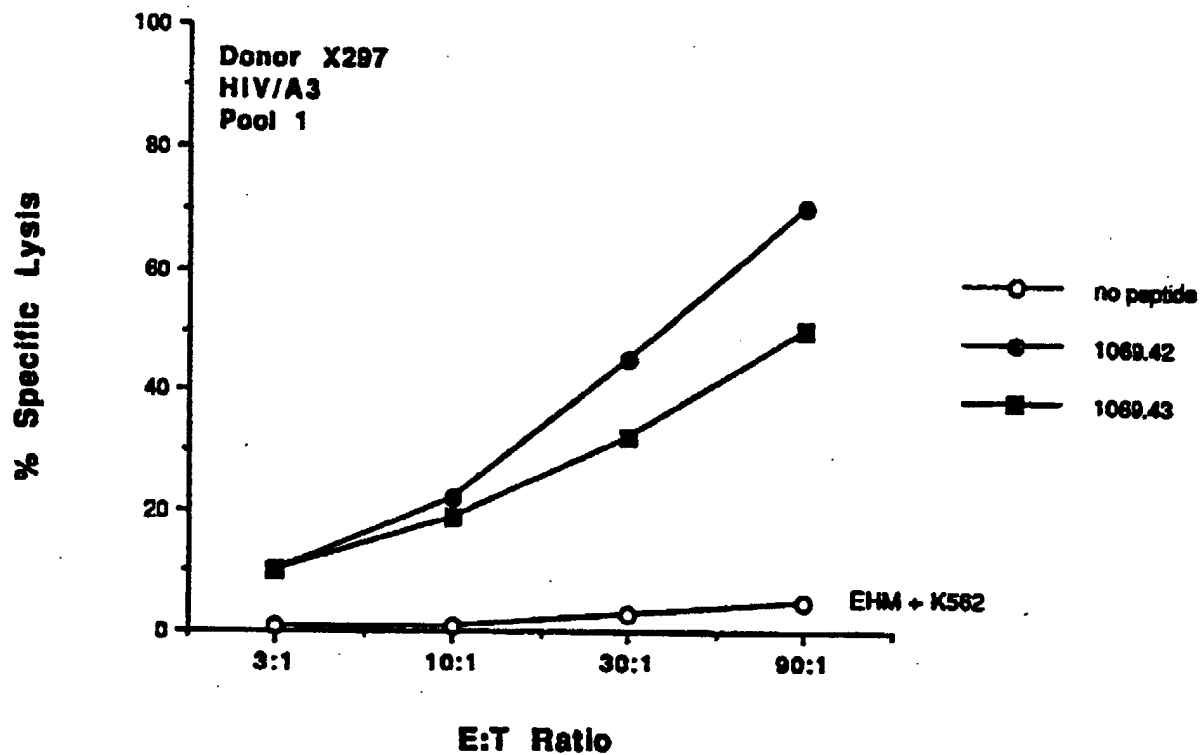
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Figure 22
Peptide Screening:
MAGE/A11



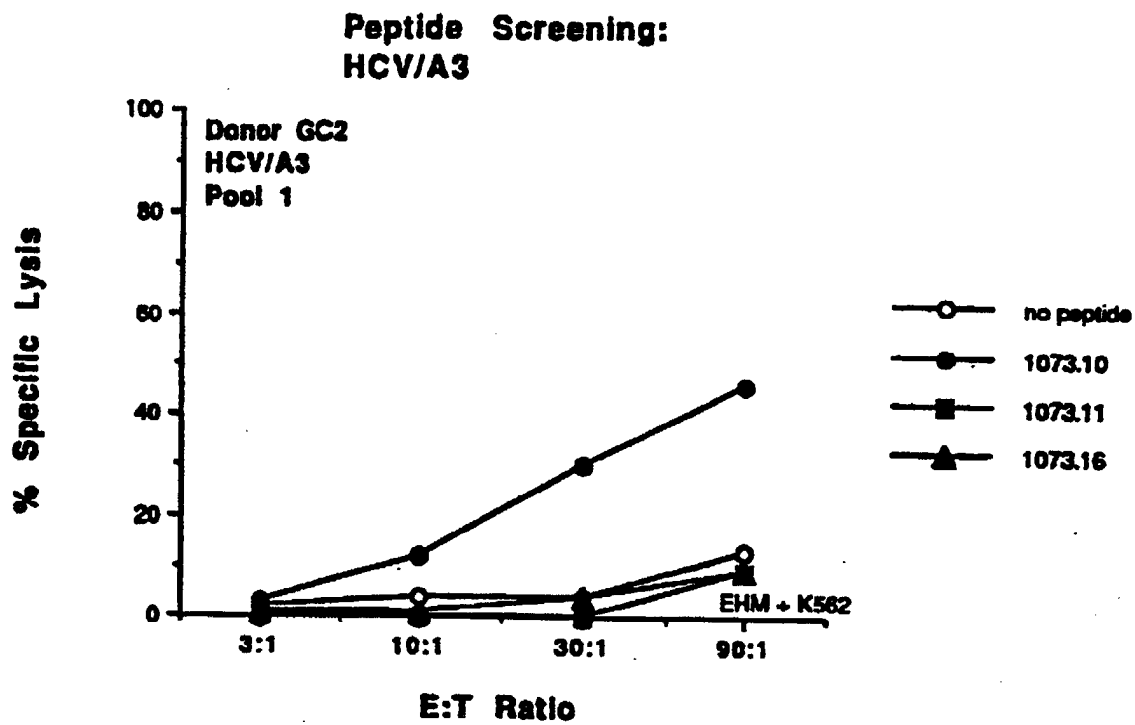
23/25

figure 23

**Peptide Screening:
HIV/A3**

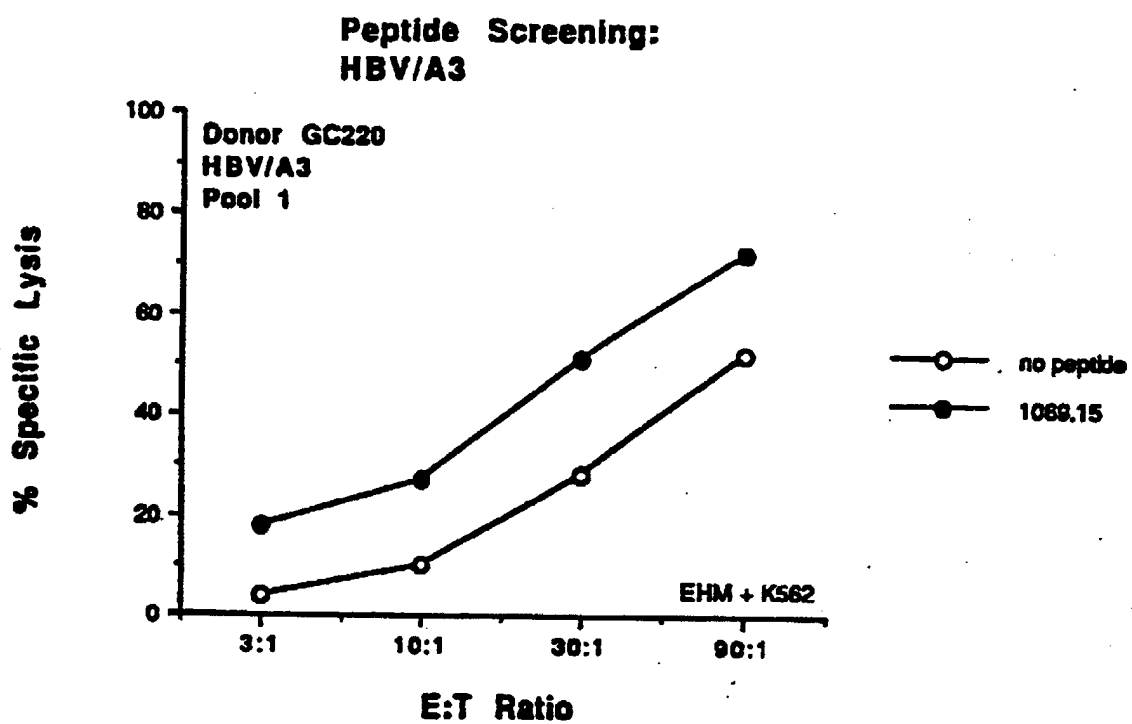
24/25

Figure 24



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Figure 25



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/07421

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 39/00; C07K 7/06, 7/08

US CL : 424/88; 514/2; 530/300, 327, 328

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88; 514/2; 530/300, 327, 328

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Exp. Med., vol. 174, number 3, issued 01 September 1991, Romero, et al., "H2K-d-restricted antigenic peptides share a simple binding motif", pages 603-612, see especially the abstract.	1-20
Y	Nature, vol. 353, issued 26 September 1991, T. S. Jardetzky, et al., "Identification of self peptides bound to purified HLA-B27", pages 326-329, see entire document.	1-20
Y	Nature, vol. 351, issued 23 May 1991, K. Falk, et al., "Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules", pages 290-296, see abstract.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A document defining the general state of the art which is not considered to be part of particular relevance	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E earlier document published on or after the international filing date	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* Z	document member of the same patent family
* O document referring to an oral disclosure, use, exhibition or other means		
* P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

01 November 1993

Date of mailing of the international search report

17 NOV 1993

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Box PCT
Washington, D.C. 20231

Authorized officer

THOMAS CUNNINGHAM

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196